Identification of human papillomavirus type 16 (HPV16) E1^E4 binding partners and the characterisation of the E1^E4/E2 interaction

A thesis submitted to the University of London for the degree of Doctor of Philosophy

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Acknowledgements

I would like to thank my supervisor John Doorbar, for always making time to answer my questions whenever the need took me and for putting up with my constant noise. The time I have spent working in lab 256, has definitely been an enlightening experience. I would like to thank all the members of the Doorbar lab past and present for all the great laughs and stimulating lab conversations that we shared as well as the practical stuff too.

In particular I would like to thank Deb Jackson, who has been invaluable for the completion of my PhD project. Pauline McIntosh, my role model in life, who became my first port of call when dealing with nasty insoluble GSTs. Clare Davy, the wise one for always reminding me to take the simplest root as a means to the end, and also for the time she dedicated for protein expression in yeast (which didn't make it to the thesis). Qian Wang, for sharing her experiences in immunoprecipation. Woei Ling Peh (senior post-doc, me just PhD), for rescuing me from several late night discussions, and giving up a lot of her time to help me to try and identify E2 expression *in vivo*. Papia Das and Pete Laskey the kiddies in the lab, whom both added to the ambience of lab 256, helping to make the time I spend doing my PhD a lot of fun.

A big thank-you goes to Ken Raj who rapidly developed into my surrogate supervisor during my PhD, and whose insightful comments definitely influenced the direction of my research. Viviane Bechtold, who arrived at the institute towards the end of my PhD but single handedly managed to extinguish my fear of quantitative RT-PCR. I would also like to thank Carlum Shiu who was my luciferase assay guru.

I am also very grateful to all those people that took the time and effort to proof read parts of my thesis, John Doorbar, Ken Raj, Viviane Bechtold, Papia Das, Woei Ling Peh, Mahmood Ayub and Clare Davy. I would also like to acknowledge our collaborators on the E2/E1^E4 project from other labs whom shared their observations with us, Helena Sterlinko Grm and Cheng-Ming Chiang.

I would like to thank my self-appointed specialist team Jonathon Hern and Woei Ling Peh (Photoshop), Louise Cheng (Formatting), big sister Shital Sorathia (Excel), little brother Suren Sorathia (detergents and statistics) and Clive Lunny (for dealing with computer stuff that I don't understand).

A warm thank-you to Steve Renwick and my other half Kathrin Scaefer (both soon to be Dr.), who have really shared the pain of thesis writing even in the adversity of their own pain/thesis.

Thanks to all the table football regulars (Keno Gutierrez, Artur Rudrigues, Justine Oyallon, Steve Renwick, Kathrin Scaefer, Pete Laskey, Mark Amoyel and Yannis Mavromatakis), climbing friends (Bill Buaas, Louise Cheng, Richard Gibbons, Wendy Leung, Clive Lunny and Vincent Hobbs) and other friends with only conventional addictions (Katie Copeland, Clare Lynch, Julia Pendred, Matt Lock, Andrea Self, Sandara Ruff, Ana Martin, Spyros Zissimopoulos and the elusive tracksuit bottom man) that gave me reasons to smile especially during my dark days in the writing cubicle.

Last but by no means least, a big thank-you goes out to my family (my mum, my dad, Shitu ben, Suface, Aashvi and Raj) for their love, encouragement and support, which I can always feel in every part of my life and in everything I do, x.

Abstract

Human papillomavirus type 16 (HPV16) is a DNA tumour virus that can infect epithelial tissue and can cause hyperproliferative lesions that can progress to cancer. The expression of the HPV late protein, E1^E4 is lost during malignant progression, but in productive infections E1^E4 is abundantly expressed in the late stages of HPV infection. The appearance of E1^E4 coincides with the onset of genome amplification and the upregulation of the viral replication proteins E1 and E2 and precedes the synthesis of the viral capsid proteins L1 and L2.

In this study we show that HPV16 E1^E4 (16E1^E4) can interact directly with several cellular and viral proteins including the late proteins L1 and L2 and the early proteins E7 and E2. HPV16 E2 associates with the phosphorylated, unphosphorylated and cleaved forms of 16E1^E4, with stable association being dependent on sequences in the central highly charged region and the extreme C-terminus of E1^E4. We observed that co-expression of E2 and E1^E4 in cultured epithelial cells resulted in the progressive accumulation of E2 to E1^E4 bound structures in the cytoplasm, and a reduction in nuclear E2 levels at late time points. This phenomenon in cells that were co-expressing E2, E1^E4 and E1, at ratios mimicking those seen in the late stages of the virus life cycle, coincided with an increase in HPV origin-dependent replication and enhanced E1/E2-mediated transcription from the wild-type HPV16 early promoter (p97).

The co-expression of E1^E4 with E1 and E2 at late time points was also shown to reduce p97 activity when E2 was expressed at levels that were optimised for E1/E2-mediated transactivation. We suggest that during productive infection, the ability of E1^E4 to bind to and alter the distribution of E2 in the cell may facilitate genome amplification and the expression of S-phase promoting HPV proteins, E6 and E7.

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Abbreviations

A_x	Absorbance at x nm
Ad	Adenovirus
Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
β-gal	β-galactosidase
bp	Base pairs
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
CBP	CREB binding protein
CIN	Cervical intraepithelial neoplasia
co-IP	Co-immunoprecipitation
COPV	Canine oral papillomavirus
CPE	Cytopathic effect
CPS	Counts per second
CREB	cAMP response element binding protein
CRPV	Cotton tail rabbit papillomavirus
DAPI	4'-6' diamidino-2-phenylindol
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide 5' triphosphates
dsDNA	Double stranded DNA
DTT	Dithiothreitol
BS	Binding sites
E4-DBP	E4 DEAD box protein
E6-AP	E6-associated protein
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EF	Elongation factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
EV	Epidermodysplasia verruciformis
FCS	Fetal calf serum
GST	Glutathione S transferase
GTP	Guanosine 5'-triphosphate
HAT	Histone acetyl transferase
HPV	Human papillomavirus
Hrs	Hours
Ig	Immunoglobulin

IRES	Internal ribosome entry site
kb	Kilobase
kDa	kilo Daltons
LB	Luria-Bertani
LCR	Long control region
LTR	Long terminal repeat
MeOH	Methanol
MHC	Major histocompatability complex
mins	Minutes
moi	Multiplicity of infection
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	Messenger ribonucleic acid
NaAc	Sodium acetate
NaAz	Sodium Azide
NEB	New England biolabs
NES	Nuclear export signal
NLS	Nuclear localization signal
NRE	Negative regulatory element
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
POD	Promyelocytic leukaemia oncogenic domain
rAd	Recombinant adenovirus
RNA	Ribonucleic acid
RNase	Ribonuclease
R/T	Room temperature
Sarkysol	N-Lauroyl scarosine
Secs	Seconds
SDS	Sodium dodecyl sulfate
TEMED	N,N,N,N'-Tetra-methyl-ethylenediamine
Tm	Melting temperature
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol
tRNA	Transfer ribonucleic acid
U	Units
URR	Upstream regulatory region
UV	Ultra-violet
VLP	Virus-like particles

1 Introduction

The Latin translation of the word virus is poison, originating from the misconception that toxins present in cell filtrates caused diseases that are now attributed to viruses. We now know that viral infections are more complex than effectively poisoning of the host. It is hoped that by increasing our understanding of viruses at the molecular level, we may identify vulnerable steps in the viral lifecycle to target for medical intervention.

1.1 Papillomaviruses

Papillomaviruses (PV) are small DNA viruses that infect stratified epithelium, and can cause a variety of hyperplasic lesions known as papillomas or warts. PV infections are highly species and tissue specific. Interest in HPV has grown significantly since it was identified as the primary etiological agent for cervical cancer in 1983 (Durst et al., 1983).

1.2 Identification of PV

The infectious nature of human warts was first demonstrated using cell-free filtrates to transmit common warts by Ciuffo *et al* in 1907 (Ciuffo, 1907). However, it was not until 1933 that the first papillomavirus, originally named Shope virus after its discoverer, but later renamed as cottontail rabbit papillomavirus (CRPV) was described (Shope and Hurst, 1933). The carcinogenic potential of CRPV was subsequently shown by experimental infection of domestic rabbits, and has since, rapidly developed as a model study for the study of viral oncogenesis (Ito and Evans C.A., 1961).

The first HPV to be characterized was isolated from a plantar wart, and was thought to be associated with the development of various benign epithelial lesions (Klung and Finch, 1965; Rowson and Mahy, 1967; Crawford, 1969). It wasn't until a different HPV type was isolated from a common hand wart almost a decade later that it was recognized that different types of HPV exist (Orth et al., 1977).

1.3 Classification

Single or multiple PV types have been isolated from the majority of studied higher vertebrates, and more recently, have also been described in some bird species. In comparison, in humans, which are the only intensively studied PV host species, over 100 PV types have been identified. PV isolates are sub-divided according to the percentage of nucleotide sequence identity that is shared between their L1 open reading frames (ORFs) (de Villiers et al., 2004).

The classification criterion used to sub-divide PV isolates was established by an international committee on the taxonomy of viruses (ICTV). This specifies that PVs which are to be described as of the same genera, must share 60 % or more identity in their L1 ORFs. Also, isolates that are to be described as of the same species (contained within a genus), must share 60-70 % L1 identity, and isolated described as PV types, (contained within species), must have 71-89 % conservation of L1 identity. PV types, which differ in their L1 ORFs by only 2-10 %, can be called subtypes and differences of less than 2 % in the L1 ORFs of PV isolates can be used to define a variant.

However, the described classification does not take into account the biological and pathological properties of each isolate. This means that phylogenically grouped viruses sometimes display divergent characteristics. For this reason, PV types may often be grouped by their species of origin, and/or their tissue tropism(s) (Howley and Lowy, 2001).

1.4 Virion structure

Infectious PV particles are ~55 nm in diameter and are composed of a single circular double-stranded DNA molecule between 7.2-8 kbp in size. The viral genome is associated with cellular histones to form a chromatin-like structure, and is encapsidated in an icosahedral protein coat composed of 72 capsomers (reviewed in (Zur Hausen, 1996) and (Howley and Lowy, 2001)). The virions themselves are non-enveloped which makes the particles relatively resistant to desiccation in the environment (Rowson and Mahy, 1967; Roden et al., 1997). The overall structural

organisation of the PV virion resembles that of murine polyomavirus and SV40 (Baker et al., 1991).

1.5 HPV transmission

Cutaneous HPV lesions can shed virus particles that are embedded in dehydrated squames, which remain stable in the environment for extended time periods. For this reason, cutaneous HPVs are typically transmitted via contaminated fomites or surfaces (Abad et al., 1994). In contrast, strong epidemiological evidence exists for the sexual transmission of many mucosal virus types (Schiffman et al., 1993; Syrjanen, 1996). In addition to this genital HPV infections can also be passed from mother to child during labour, resulting in laryngeal infections (Lowy and Howley, 2001).

In comparison with cutaneous HPV types, mucosal lesions are less productive, and virions are normally shed into mucosal secretions of the genital tract, this limits their propensity to spread via objects (Abad et al., 1994). However, the resistance of some mucosal PVs to desiccation, and the detection of genital infections in non-sexually active individuals, suggests that non-sexual transmission of genital HPV types is possible (Abad et al., 1994).

Epidemiological studies of some clinically important genital HPV types i.e. HPV6, 11 and 16, suggest that HPV infections are more prevalent in men than in women, even though women more commonly display symptoms of infection (van Doornum et al., 1992). In addition, many HPV infections in the general population are thought to be silent or latent, and thus can only be detected easily in immunocompromised individuals. A recent study of HPV infections in the immunocompromised, suggests the prevalence of HPV infection in the general population may be as high as 60-80 % (Broker et al., 2001).

Novel HPV isolates and also HPV types that were previously thought to be rare have frequently been identified in immunocompromised individuals. Whereas, clinically important HPVs, that are considered to be common pathogens in the general population, are less prevalent. Together this suggests that some clinically innocuous

HPV types may be present in the human microflora, and so only behave as opportunistic pathogens (Broker et al., 2001).

1.6 HPV infection

HPV Infection is thought to occur via micro-wounds produced by skin trauma. These provide a route for the virus through the layers of dead and dying cells of the epithelium to the basal layer. HPV must infect cells in the basal layer in order to persist, as these are the only mitotically active cells in the epithelium. Infection of suprabasal cells can also occur, but this only causes transient infections that can be resolved by epithelial turnover (Vinther and Norrild, 2003).

The α6 integrin receptor was initially identified as the candidate cellular receptor for HPV infection (Evander et al., 1997). Subsequently, *in vitro* receptor-binding studies, have demonstrated that not all PVs have a requirement for α6 integrin (Giroglou et al., 2001a; Shafti-Keramat et al., 2003). Heparan sulfate proteoglycans (HSPGs) have been proposed as primary attachment molecules for many viruses and more recently also for PV (Joyce et al., 1999).

HSPGs are found on the surface of most cells and in the extra cellular space (Oksala et al., 1995). The C-terminal domains of several HPV L1 proteins display similarities to known heparin-binding partners (Joyce et al., 1999). In addition, positively charged L1 and L2 peptides can compete with VLPs for HSPG association. Providing further evidence in support of the hypothesis, that HSPGs may act as cellular receptors for PV cell entry (Bousarghin et al., 2004).

Several HSPGs can serve as PV receptors. The HSPG, syndecan-1 has been proposed as the candidate receptor for natural HPV infections (Shafti-Keramat et al., 2003). Syndecan-1 is normally present on the surface of suprabasal cells, but expression is only switched on in basal cells during wound healing (Oksala et al., 1995). Following receptor binding virus particles are internalized by clathrin- or caveolae-mediated endocytosis into the cell, before being transported to the nucleus (Giroglou et al., 2001a). PVs despite their strict tissue tropisms have the ability to bind and enter a

wide range of non-permissive cell types. This suggests that this property of the virus is not restricted at the level of virus entry, but is more likely regulated by events downstream (Chiang et al., 1992; Del Vecchio et al., 1992).

1.7 Diseases associated with papillomaviruses

The pathology of PV is intricately linked to the differentiation program of the epithelium. Thus, a basic knowledge of the epithelial tissue is required, to be able to understand PV infections.

1.7.1 The epithelium

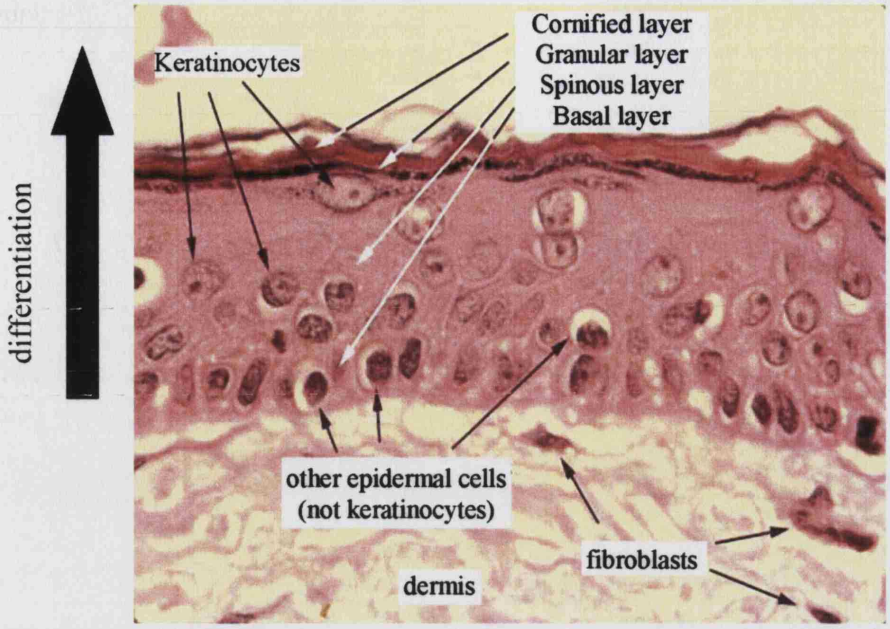
The epithelium (*epi*, on + *thelium*, surface) covers body surfaces, forming the skin or cutaneous epithelium, and also lines the oral and anogenital cavities forming the mucosal epithelium. The epithelium (Figure 1.1a and b) consists of flattened (squamous) cell layers overlying several cell strata, each of which displays morphological properties that distinguish it from the adjacent layers. The principal cell type found in the epithelium is the keratinocyte, but in addition Langerhan cells which are involved in immuno-surveillance, and Merkel and dendritic cells of the nervous system are also present. In cutaneous epithelia, melanocytes, which are cells involved in pigment production are also found (King, 2003).

Epithelial tissue can be thought of as polarized, with an apical surface (i.e. the cornified or superficial layer), from where cells are sloughed off, and an attached surface (i.e. the basal layer), where cell proliferation occurs (Walker et al., 2003). These epithelial surfaces are linked by a gradient of differentiating cells. Differentiation is a process by which cells undergo changes in morphology, and function during their life span (Fuchs, 1993).

Keratins are the most abundant protein present in both differentiated and undifferentiated keratinocytes. These proteins are α-helical, and can assemble into intermediate filament (IF) networks. In the basal cells of both cutaneous and mucosal epithelium, keratins K5 and K14 are expressed. As these cells differentiate, K5 and

Figure 1.1 Squamous epithelium
(a) A hematoxylin and eosin (H&E) stain of a cross section of human skin (adapted from http://www.siumed.edu/%7Edking2/intro/skin.htm#epidermis with the permission of Prof. David King, Southern Illinois University, Illinois, USA). Different cell types and epithelial layers are labelled. (b) Table distinguishing between the nomenclature of cutaneous and mucosal epithelium and briefly outlining the characteristics of the different epithelial layers. (c) H&E stained cross section of a cervical biopsy, displaying both normal and dysplastic epithelial phenotypes. The basal layer of the epithelium is denoted by the white line.

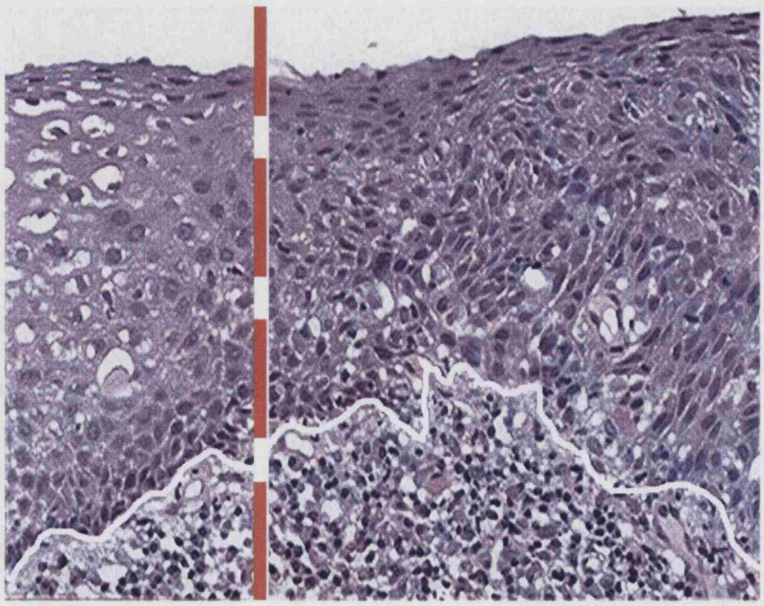
a



b

Cutaneous epithelium layers	Mucosal epithelium layers	Characteristics
Cornified layer	Superficial layer	Dead keratinized flattened cells many of which have lost their nuclei. The cornified layer is a lot thicker than the superficial layer
Granular layer		Cells metabolic activity decreased. Ceramide, sterol and fatty acid content of the plasma membrane increased. Keratohyalin (darkly stained granules) containing loricrin and profilaggrin deposits.
	Intermediate layer	Cells metabolic activity decreased. Ceramide, sterol and fatty acid content of the plasma membrane increased. Keratin filament bundles are produced.
Spinous layer	Parabasal layer	Post-mitotic but metabolically active cells attached to one another by desmosomes. Differentiation specific keratins are synthesised.
Basal layer	Basal layer	Mitotic cuboidal cells are attached to basement membrane by hemidesmosomes

c



Normal cervical epithelia

High grade dysplasia

K14 are down regulated, and differentiation-specific keratins are produced. These are keratins, K1/K10 and K4/K13 in cutaneous and mucosal epithelia respectively (reviewed in (Fuchs and Weber, 1994; Chow and Broker, 1997)).

The differentiated cells in the granular or superficial layer of the epithelium form a resistant barrier to the outside. In these cells the keratin cytoskeleton is bundled together by filaggrin, to form anchors for proteins of the cornified cell envelope (CCE), which is itself deposited on the cytoplasmic face of the plasma membrane. The major constituent of the CCE is loricrin, but other components which are present include, involucrin and 'small proline-rich' proteins. These proteins are cross-linked to each and the plasma membrane by isodipeptide and disulfide bonds (Fuche and Byrne, 1994).

1.7.2 Papillomas

Papillomas are benign overt epithelial tumours that arise following a PV infection. Lesions may develop on any stratified squamous epithelium, and are called warts when they are produced on the skin and condylomas when they are present at genital sites. Most papillomas if left untreated will spontaneously regress after months or years of persistence. However, in a small number of cases associated with certain types of human and animal PVs, infections may gradually become malignant (reviewed in (Lowy and Howley, 2001)).

High-grade dysplasia (Figure 1.1c) and carcinomas are thought to arise from abortive PV infections (Middleton, 2003). In which progeny viruses are no longer produced but genetically active viral DNA persists. However, malignant progression is not an inevitable outcome of infection, as lesions may regress at any stage before the development of invasive cancer. Although the likelihood of regression is inversely related to the stage of dysplasia (Lowy and Howley, 2001).

1.7.3 Pathology of animal papillomaviruses

PVs have been isolated from many vertebrates. The biology of some of these animal PVs, which have been isolated from rabbits (CRPV and rabbit oral PV (ROPV)),

cattle (BPV1, 2 and 4) and dogs (canine oral PV (COPV) have been developed as experimental models for the study of PV infections.

CRPV causes benign productive lesions in its natural host, which may regress, persist or less frequently may develop into cancers. The risk of progression of CRPV infections into carcinomas is increased in experimentally infected domestic rabbits, in comparison with CRPV infections of cotton tail rabbits. Of the PV types studied, only BPV1 and 2 have demonstrated the ability to infect both fibroblasts and keratinocytes, although lesions are only productive in the latter cell type. In addition, both BPV types exhibit a broad experimental host range, causing disease in hamsters and horses as well as cattle (reviewed in (Lancaster and Olson, 1985)).

BPV4 is most commonly associated with papillomatosis of the alimentary canal of cattle. High cancer incidence is found in cattle that graze in areas infested with bracken fern (Jarrett, 1978; Smith et al., 1994). Bracken fern contains the carcinogenic compounds, ptaquiloside and quercetin, and like tar, bracken fern has been shown to act as a co-carcinogen in some PV-related malignancies (Rogers and Rous, 1951; Jarrett, 1978; Campo, 2002).

1.7.4 The pathology of human papillomaviruses

1.7.4.1 Non-malignant HPV infections

HPV types generally are more successful in either cutaneous or mucosal epithelium, but exceptions exist, such as HPV2, 27 and 57 which can infect both epithelial types well. The vast majority of HPV infections display no clinical symptoms, and instead, cause in-apparent micro-lesions. However, when lesions are evident, it seems that different HPV types tend to be predominant at specific anatomical locations. For example, HPV11 is most commonly associated with benign external genitalia condylomas (genital warts), but although less frequently, it is also associated with infections of the oral mucosa, and the larynx.

1.7.4.2 Malignant HPV infections

Cervical cancer is the second most prevalent cancer worldwide, and is the fifth leading cause of cancer deaths in women (Longworth and Laimins, 2004b). In a study of almost 1 000 cervical cancers, HPV DNA was detected in 99.7 % of specimens, of which 49.9 % contained HPV16 DNA (Walboomers et al., 1999). HPV16 is thus considered a "high-risk" virus type, and is the most significant determinant for malignant progression, in HPV aetiology (Lorincz et al., 1992; IARC, 1995). Yet still less than 5 % of HPV16-infected individuals, develop cancer.

Other mucosal HPVs have also been categorized as high-risk viruses, these include, HPV18, 39, 45, 31, 33 and 35 (Chow and Broker, 1997). This classification was originally based on the frequency at which the HPV isolates were found in cervical and anogenital cancers, but more recently, the definition has been extended, to incorporate biological properties of the virus types (zur Hausen, 1985). For example, the ability of high-risk HPVs to immortalize human keratinocytes (Pirisi et al., 1987; Dyson et al., 1989; Werness et al., 1990; Duensing et al., 2000). Low-risk viruses such as HPV6 and 11, usually cause benign papillomas, and are rarely found in cervical and anogenital cancers, unless co-carcinogens or somatic mutations in tumour suppressor genes act as co-factors for malignant progression (reviewed in (Zur Hausen, 1996)).

The first link between HPV infection and cancer development came from patients with epidermodysplasia verruciformis (EV) (Orth et al., 1980). This is a rare hereditary condition characterized by susceptibility to cutaneous HPV infections (Orth et al., 1978). It has been estimated that in EV patients, HPV-lesions at light-exposed sites will progress to non-melanoma skin cancers (NMSCs) in 50 % of individuals (Jablonska and Majewski, 1994). More than 20 different HPV types have been identified in benign EV lesions. In contrast, in malignant lesions HPV types, 5 and 8 prevail, with other HPVs only rarely being found. HPV DNA is also present in the majority of examined NMSCs that occur in the general population. However, the role of HPV in NMSCs in non-EV patients, remains to be defined (Jablonska and Majewski, 1994; Forslund et al., 2003).

HPVs are also associated with epithelial lesions of the aerodigestive tract and some rare respiratory cancers (reviewed in (Lowy and Howley, 2001)). The latter association is supported by the identification of HPV DNA, most commonly HPV type 16, in oral, larynx, nasal and oesophagus cancers (IARC, 1995). In aerodigestive and possibly also junctival and bladder cancers, HPVs are thought to act as co-carcinogens as opposed to major determinants of malignancy. This is because HPV DNA is not consistently detected in these tumours like it is in cervical cancers. Although, this may simply be a reflection of probe limitations, as in most studies only anogenital HPV types are screened for.

1.8 Malignant progression

The deregulation of the viral E6 and E7 genes of high-risk HPVs is an important step in the development of cervical cancer (Schwarz et al., 1985; Jeon and Lambert, 1995). The expression of these viral oncogenes in the basal layer has been shown to lead to chromosomal instability, which it is speculated, favours the integration of the viral DNA into the cellular genome. HPV integration results in either the loss of the E2/E4 open reading frames (ORFs) or less frequently, the E1 ORF (Durst et al., 1985; Duensing et al., 2000; Melsheimer et al., 2004). These events are thought to lead to uncontrolled cellular proliferation, due to the loss of viral transcription factors (TFs) that regulate E6 and E7 transcription (Cripe et al., 1987; Durst et al., 1991; Romanczuk et al., 1991).

Accumulated evidence supports the proposed mechanism of HPV-induced carcinogenesis. For example, in benign lesions and in low and intermediate stages of cervical intraepithelial neoplasia (CIN I and CIN II respectively), viral genomes have been shown to be exclusively episomal (Schwarz et al., 1985; Shirasawa et al., 1986). In contrast, in high grade lesions (CIN III) and in cervical cancers, integration of the HPV DNA into the host chromosome was common, and was frequently associated with a selective growth advantage (Choo et al., 1987; Jeon et al., 1995).

However, a recent study by Pertsaro *et al*, has modified our previous understanding of HPV cancer prognosis, by showing that HPV integration can occur earlier in HPV

pathogenesis than was previously thought. In this study, malignant and non-malignant cervical biopsies infected with HPV16 were analysed, using a real-time PCR method to detect episomal and integrated viral DNA. This sensitive technique suggested that integration of the viral genome had already occurred in some pre-CIN, CIN I and II lesions as well as in CIN III and cervical cancer biopsies. Markers of oncogenic progression were also quantified in this study. In the majority of progressive lesions that were examined, high ratios of integrated to episomal HPV DNA was found, which could also be correlated with high E6/E7 mRNA levels (Peitsaro et al., 2002). RT-PCR and *in-situ* hybridization studies of CINs and cervical cancers also support the role of E6 and E7 in carcinogenesis, as transcripts encoding these viral oncogenes have been shown to be incrementally upregulated in parallel with disease progression (Durst et al., 1992a; Nakagawa et al., 2000).

The extent of HPV genome integration that occurs is obviously important for the transformation of many HPV lesions. However, a recent study has shown that HPV16 DNA was found to be exclusively integrated in only 50 % of the examined cervical cancers (Hudelist et al., 2004). This suggests in a large proportion of these cases, that other factors contribute to the malignant phenotype. It has been proposed that sometimes abnormalities in the expression of cellular tumour suppressor, and growth stimulating genes, may compensate for inadequate E6 and E7 levels, to bring about transformation (reviewed in (Wolf and Ramirez, 2001)).

Chromosomal abnormalities may be induced by the viral genome integration event itself (Segawa et al., 1999). HPV frequently integrates at common fragile sites (CFSs), which are chromosomal loci that tend to form breaks and gaps (Chung et al., 1992). Integration, can lead to the loss of heterozygosity (LOH) of genes, which are present at CFSs (Thorland et al., 2000). LOH at segment 3p14 occurs in 70 % of primary cervical cancers, and in 30-57 % of CINs, as a result of HPV genome integration (Larson et al., 1994; Wilke et al., 1996; Chu et al., 1998). This region overlaps with the CFS locus, FRA3B and contains the fragile histidine triad (*FHIT*) gene (3p14.2). It is possible that *FHIT* disruption could contribute to malignant progression, as it is a known tumour suppressor (Thorland et al., 2000).

LOH of *FHIT* specifically, has been shown in several cancers, but its significance in cervical cancer development has not been resolved (Ohta et al., 1996; Baffa et al., 2000; Ingvarsson, 2001; Pylkkanen et al., 2002; Sukosd et al., 2003). A study by Chu *et al*, shows that deletions and insertions in the *FHIT* gene are common in abnormal cervical tissue, but also occur in normal cervical tissue and so cannot be attributed to HPV integration (Chu et al., 1998). However, more recent studies have demonstrated that *FHIT* transcripts are aberrant or reduced, and that FHIT protein expression is absent, in cervical cancers, and cervical cancer cell lines (Segawa et al., 1999; Connolly et al., 2000). These findings suggest that loss of the *FHIT* gene may promote cervical cancer development.

1.9 Cervical cancer prevention

1.9.1 Cervical Screening

Carcinoma of the cervix accounts for ~12 % of all female cancers worldwide, and causes ~250,000 deaths annually (Ferlay et al., 2001). Regular screening of the cytology of the cervix using the Pap smear, has significantly reduced both the incidence and mortality from cervical cancer in Western countries (Vizcaino et al., 2000). The increased understanding of the molecular pathogenesis of cervical cancer also offers the opportunity of reducing risk further by increasing accuracy of screening by incorporating viral and cellular biomarkers into prevention programmes (Williams et al., 1998; Freeman et al., 1999; Keating et al., 2001; Middleton, 2003). Testing for HPV late gene expression, in combination with surrogate markers of E6 and E7 expression, would potentially allow accurate distinctions to be made between high and low grade CINs. Also, the use of type-specific HPV antibodies in follow up tests, could be used to help predict the likely-hood of malignant progression of high-risk HPV infections (Middleton, 2003).

In developing countries cervical cancer is the leading cause of female cancer mortality. Organized screening is not in place in many of these countries largely due to the associated costs. This means ~80 % of affected women are not identified until the disease has reached an advanced stage, and is no longer regarded as curable

(Baldwin et al., 2003; Frazer, 2004). Screening techniques which are lower in unit cost such as visual inspection using acetic acid (VIA), and the prospective prophylactic vaccine currently under-going clinical trials, could have an impact in reducing mortality particularly in developing countries (Blumenthal et al., 2001).

1.9.2 HPV vaccination

Persistent infection with high-risk HPV types is the most important risk factor leading to the development of cervical cancer (Zur Hausen, 1996). Together the two high-risk genotypes HPV16 and HPV18 account for ~70 % of cervical cancers (Munoz et al., 2003). Vaccination studies in animals have shown that protection against PV-associated disease is genotype specific, and for this reason human vaccines are being developed that are targeted against HPV16 and 18 (Breitburd et al., 1995; Suzich et al., 1995; Giroglou et al., 2001b).

Two types of vaccine are currently under development for HPV infection. One is prophylactic, and the other is therapeutic (reviewed in (Frazer, 2004)). Prophylactic vaccines induce virus-neutralizing antibodies, and can also prime the adaptive immune system. Thus potentially being able to reduce the number of HPV positive cells post infection, and also by being able to stimulate the production of memory cells, which would protect against future viral challenges. In contrast, the therapeutic vaccine would induce a cell-mediated response by activating killer T-cells. This should reduce the size of the HPV-infected cell population, but would not provide long-term immunity to any real extent because helper T-cells and thus the humoral immune system would not be primed.

In HPV infected humans and in animals, neutralizing antibodies to the major capsid protein, L1 are found in 50-100 % of individuals (Combita et al., 2002a; Combita et al., 2002b). For this reason L1 is the antigen that has been used for the development of the prophylactic vaccine. The E7 protein, and to a lesser extent the E2 protein, are the subject of therapeutic vaccine research, as they can induce protective cellular immunity (McGarvie et al., 1995; Selvakumar et al., 1995). The other viral ORFs

have not been shown to induce detectable specific immune responses (reviewed in (Breitburd and Coursaget, 1999)).

L1 virus-like particles (VLPs) produced *in vitro* structurally mimic the virus. VLPs, elicit sufficient systemic and mucosal immunity so that persistent, high-risk infections are prevented (Kirnbauer et al., 1996; Roden et al., 1996). The potential benefits of HPV vaccination are huge, but eradication of the disease is far more complex. A concern would be that successful vaccination against HPV16 and HPV18 could potentially cause minor high-risk viruses to become more prevalent, due to a lack of competition by HPV16 and HPV18. To combat these potential problems new vaccines may continually need to be designed to shadow disease evolution.

1.10 The study of HPV infections in the laboratory

The strict tissue/species tropism of HPV and its requirement for differentiating epithelium to complete its life cycle creates innate difficulties for the production of these viruses in the laboratory. PV animal models have been useful for lifecycle studies, and in particular for the study of papilloma regression, but host restrictions prevent the examination of HPV in these systems.

Xenotransplantation model systems have been developed which enable the completion of the HPV life cycle and the production of infectious virions in host tissue. This is achieved by grafting HPV infected human foreskin under the renal capsule of athymic mice or onto the back of severe combined immuno-deficient (SCID) mice. Xenotransplantation has been used to study the life cycle of mucosal HPVs (Kreider et al., 1987; Brandsma et al., 1995; Bonnez et al., 1998).

Monolayer cells and VLPs, have been used together to study early events of HPV infection (Zhou et al., 1991; Muller et al., 1995). Additionally, crude methods for inducing differentiation of monolayer cells such as methylcellulose treatment of keratinocytes have been of value in the characterization of the productive stage of the virus life cycle. However, in this system the viral capsid proteins are not consistently

expressed, which makes it difficult to study the late stages of infection (Ruesch et al., 1998).

Another *in vitro* method for the reconstruction of the HPV life cycle is epithelial raft culture, which has proved to be particularly valuable for the study of viral gene function. This is because raft culture enables the consequences of experimentally mutated HPV genomes to be examined in the context of the virus life cycle. Basal keratinocytes that have been transfected with HPV viral genomes are seeded onto a dermal membrane equivalent, consisting of a collagen gel matrix and fibroblast cells. The cells are grown until confluent and then the culture, supported on the collagen matrix is raised to the tissue culture medium-air interface and allowed to differentiate. The keratinocytes stratify and exhibit a differentiated morphology enabling completion of the HPV life cycle *in vitro* (Dollard et al., 1992; Flores et al., 1999).

1.11 Papillomavirus genomic organisation

Different PV types have similar genomic organisation (Figure 1.2), with ORFs that are well conserved in terms of size and location. All of the PV ORFs are encoded on one strand of the DNA, and overlap with each other to varying extents. The coding strand of most known PVs contain 8 translated ORFs, however, some animal PVs such as BPV1, encode up to 10 genes. PV genes can be split into two groups, early (E), and late (L), depending on their location within the genome (Howley and Lowy, 2001). The transcription of these genes is under the control of at least four promoters, the early promoter regulates mainly the transcription of E6 and E7 genes, and the late, or differentiation-dependent promoter controls the transcription of viral replication, and capsid genes (Ozbun and Meyers, 1998b).

The set of proteins that are expressed from transcripts derived from the late promoter depends to an extent upon the usage of different polyadenylation sequences, located at the end of the E5 and L1 ORFs (Schwartz, 2000). The early and late genes are spatially separated in the genome by a non-coding region of DNA, which is called the long control region (LCR), or the upstream regulatory region (URR). This contains

the origin of DNA replication, an epithelial-specific enhancer element and the early promoter (Figure 1.3).

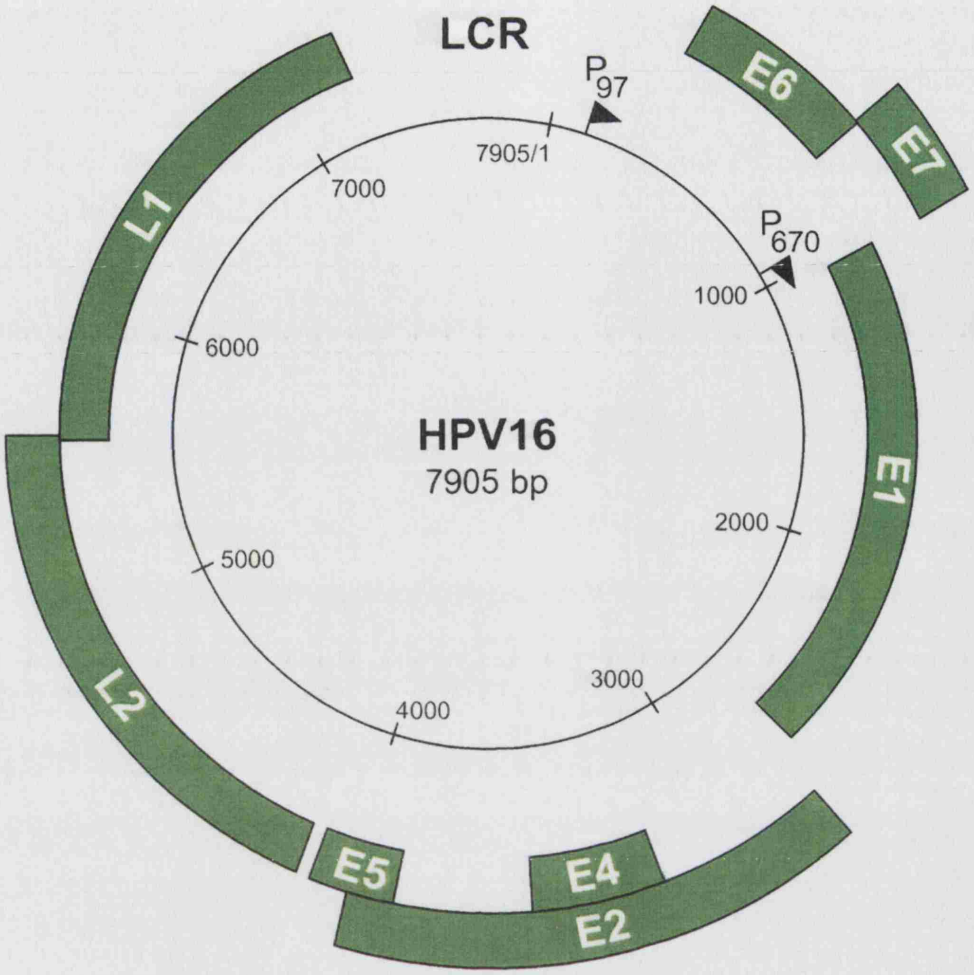


Figure 1.2 Genomic map of the HPV16 genome
The HPV16 genome consists of a double stranded DNA molecule that encodes 8 genes as labelled. The two major promoters which regulate viral gene transcription are the early promoter, p97 and the late promoter, p670 which are named according to their nucleotide position. The LCR is a non-coding region of the genome that contains sequences that regulate the expression of early genes.

Figure 1.3 Schematic representation of the HPV16 LCR
Elements contained within the LCR are indicated on the diagram, and the nucleotide positions of some of these are specified. The relative positions of the cis elements to the regions of the LCR i.e. origin of replication (ori) and transcriptional enhancer are also shown. Open boxes represent the HPV ORFs which overlap with the LCR and are labelled. Coloured circles represent transcription factor binding sites. The positive and negative symbols shown above three of the E2 BSs indicate the effect of E2 occupancy of these sites on transcription.

1.12 Overview of the PV life cycle

In the basal layer of the epithelium the PV establishes itself as a low copy number nuclear plasmid (estimated to be at 10-200 copies/cell), by replicating the viral genome usually once per cell cycle (Stanley et al., 1989; De Geest et al., 1993). This can be described as the non-productive stage of the life cycle, and is thought to be important in providing a reservoir of genomes for latent infections. As infected cells divide, daughter cells are produced, a subset of which migrate up towards the epithelial surface. The PV E6 and E7 proteins are the first viral genes to be expressed in the migrating cells. Together these proteins promote S-phase entry of the cells in which they are expressed, by directly targeting cell cycle regulators. The most important targets of E6 and E7 respectively, are p53 and the retinoblastoma susceptibility protein (RB).

Viral genome amplification requires supercoiled HPV DNA templates, ATP, cellular replication factors (e.g. DNA polymerases α and δ, replication protein A (RPA), proliferating cell nuclear antigen (PCNA), replication factor C (RFC) and toperisomerases I and II) and viral replication proteins (E1, E2, E1^E4 and E5) (Breitburd et al., 1987; Ustav and Stenlund, 1991; Ustav et al., 1991; Yang et al., 1991; Hughes and Romanos, 1993; Kuo et al., 1994; Doorbar et al., 1997; Fehrmann et al., 2003; Genther et al., 2003). This stage of the life cycle tends to be concentrated in the intermediate layer of the epithelium in mucosal HPV infections. By comparison, the evolutionary distinct cutaneous HPVs tend to amplify their DNA for a larger proportion of the life cycle, indeed genome amplification in these lesions usually extends over three epithelial layers (parabasal, spinous and granular) (Peh et al., 2002).

The initiation of viral replication must be coordinated with the activation of cellular replication machinery. The timing of cellular replication is controlled by cyclin-cdk (cyclin-dependent kinase) complexes, which modulate the function of key regulatory proteins, by phosphorylation. The PV E1 protein is thought to also utilise this cellular regulatory mechanism. E1 can be phophorylated on its N-terminal domain by several kinases, including cyclin-cdk complexes, the latter is essential for determining the

nuclear localisation of E1 prior to replication (Zanardi et al., 1997; Ma et al., 1999; Deng et al., 2004).

PV DNA can be replicated in two ways, theta replication and rolling circle replication. Theta mode requires an initiation event to occur to produce each daughter genome, whereas the rolling circle mode requires only one initiation event to produce multiple DNA molecules, and is comparatively more efficient (Flores and Lambert, 1997). There is evidence that suggests the virus uses the theta mode of replication for genome maintenance, and switches to the rolling circle mode for vegetative genome amplification (Dasgupta et al., 1992; Flores and Lambert, 1997). The PV life cycle is completed in the upper layers of the epithelium, where the viral genome is packaged into icosahedral capsid coats composed of L1 and L2 proteins. Subsequently, infectious virions are shed from the surface of the epithelium.

1.13 HPV proteins

This following section describes in greater depth the biological characteristics and functions of HPV proteins in the life cycle (summarized in the previous section), and also where appropriate in malignant progression. As this study is focused on HPV16 protein-protein interactions, particular attention is given where possible to reporting the properties of this virus type and that of other high-risk viruses.

1.13.1 The E6 protein

E6 is a dimeric protein with two zinc finger domains (Barbosa et al., 1989; Grossman and Laimins, 1989; Stacey et al., 1994). The E6 protein is thought to be expressed in the basal and parabasal layers of the epithelium of productive lesions, and intermediate layers of the epithelium, in HPV-associated neoplasia (Durst et al., 1991). HPV16 and other high-risk HPVs have been shown to produce three truncated E6 splice variants, E6*I, E6*II and E6*III in addition to the full length E6 protein (reviewed in (Myers and Androphy, 1995)). It has been suggested that E6 splice variants favour the translation of the E7 ORF, following slippage of the ribosome

from the E6 start codon to the E7 start codon during translation (Smotkin et al., 1989; Stacey et al., 1995).

1.13.1.1 Functions of E6

The role of the E6 protein in the development and maintenance of the malignant phenotype has been well characterised but in contrast, the role of E6 in the virus life cycle is not as well understood.

Replication

E6 proteins of both high- and low- risk viruses can associate with the product of the tumour suppressor gene *p53*, but only E6 proteins from high-risk viruses direct p53 degradation (Scheffner et al., 1990; Werness et al., 1990; Crook et al., 1991; Li and Coffino, 1996). The main function of the p53 protein is to modulate the transcription of genes which are involved in cell cycle arrest and apoptosis (Hofseth et al., 2004). E6 abrogates p53 function by promoting its degradation, and to a lesser extent, by retaining p53 in the cytoplasm (Scheffner et al., 1990; Mantovani and Banks, 1999). The latter mechanism prevents p53 translocation into the nucleus, which is required for it to act as a transcription factor (TF). E6-mediated degradation of p53 is via the ubiquitin-proteasome pathway, and requires the formation of a ternary complex between E6, p53 and the E6 associated protein (E6-AP), which is an E3 ubiquitin ligase (Scheffner et al., 1993).

It has been shown that the intrinsic activity of the p53 protein can inhibit the transient amplification of an HPV origin-containing plasmid in a concentration-dependent manner, whereas, maintenance, once-per-cell cycle replication is not sensitive to p53 (Lepik et al., 1998; Ilves et al., 2003). The mechanism by which p53 inhibits origin-dependent amplification is not known, but it has been suggested that p53 is likely to inhibit a function of rolling circle replication but not theta replication, as these different methods are employed for amplification and maintenance replication of PVs, respectively (Flores and Lambert, 1997). The down-regulation of p53 may thus be required to enable efficient viral genome amplification to occur.

Apoptosis of cells can occur in a p53-independent manner, which involves the activation of members of the Bcl-2 pro-death protein family (Hofseth et al., 2004). The E6 protein from mucosal HPVs can prevent p53-independent apoptosis, as well as p53 dependent-apoptosis, by binding to and degrading members of the Bcl-2 family, Bak and Bax (Magal et al., 2005). In support of this it has also been shown that the Bak protein which is abundantly expressed in the upper layers of normal epithelium is significantly reduced in colorectal carcinomas (Krajewska et al., 1996). It should be noted that E6-mediated Bak degradation is not as efficiently achieved by E6 proteins from low-risk HPVs, in comparison with E6 proteins from high-risk HPVs. The differential ability of the E6 proteins of individual HPVs to circumvent apoptosis is thought to be fundamental to the oncogenic potential of the virus-type.

Transcription

HPV16 E6 and the E6*I proteins can transactivate the HPV16 early promoter (p97), when expressed at low levels, but at high concentrations the full-length E6 protein can also repress transcription of the E7 ORF (Shirasawa et al., 1994). E6 and E6 splice variants can associate with an E6-inducible enhancer (IE6) sequence, located in the LCR. This element has a relatively weak effect on early transcription, in comparison with the downstream E2-inducible element, and the cellular enhancer site. However, it has been hypothesised that E6 and E6*I may contribute to the tight transcriptional control of E6 and E6 variants in latent HPV infections, and also to the upregulation of E6 proteins in malignant cells (Gius et al., 1988; Shirasawa et al., 1994). It is also possible that the association of E6 and E6 variants with cellular transcription factors may contribute to the modulation of viral transcription. This is supported by findings that show E6 binding can abrogate proteins that have been shown to promote p97 transactivation, e.g. p53, interferon regulatory factor 3 (IRF-3), and also members of the p300/CREB-binding protein (CBP) family of histone acetyltransferases (HATs) (Ronco et al., 1998; Patel et al., 1999).

It has recently been shown that E6 and E6-AP together are essential for mediating the global effects of E6 on cellular transcription targets (Kelley et al., 2005). Cellular genes that are targets for high-risk E6 transcriptional upregulation are thought to influence the immortalized phenotype. A well characterised example is the human

telomerase catalytic subunit (*hTERT*). Telomerase is an enzyme which is normally only active in germ cells, where it is important for the maintenance of DNA repeats located at the end of chromosomes, which are called telomeres (Kim et al., 1994). Telomeres in somatic cells are shortened following each cell division, as the extreme 3'-ends of chromosomes are not normally replicated. When telomeric length reaches a critical point, chromosome attachment to spindle bodies cannot occur efficiently, resulting in mitotic arrest, and subsequent cell death following the activation of the DNA damage response pathway (Cooke and Smith, 1986).

E6-induced telomerase activity allows telomeres to be maintained at a length that is sufficient for cell survival, and this ultimately increases the number of divisions/cell (Counter et al., 1992; Klingelhutz et al., 1996). Telomerase activation is thought to occur as a late, but common, event in the process of HPV16 immortalization, but most studies examining cervical tissue indicate that significant telomerase activation does not occur until lesions have progressed to CINs, suggesting that the ability of E6 to activate telomerase is only important in the context of cancer (Snijders et al., 1998; Nowak, 2000). E6 expression has also been shown to increase transcription of vascular endothelial growth factor (VEGF), c-Myc the transforming growth factor-beta (TGF-β). Once again the roles of these proteins in tumourigenesis have been well documented but their relevance to the virus life cycle has not been adequately addressed (Dey et al., 1997; Kinoshita et al., 1997).

Maintenance

The E6 proteins from high-risk HPVs have been shown to interact with proteins that harbour multiple copies of a protein-protein interaction domain, called PDZ (PSD-95/Discs Large/ZO-1), via a motif in the E6 C-terminal domain (Kiyono et al., 1997). The E6 protein has been shown to interact and induce the degradation of PDZ domain-containing proteins such as hScrib, MAG-1, MAG-2, MAG-3 and MUPP1 *in vitro* via the ubiquitin-proteasome pathway (Gardiol et al., 1999; Glaunsinger et al., 2000; Lee et al., 2000; Nakagawa and Huibregtse, 2000; Pim et al., 2000; Thomas et al., 2002). Several of these proteins are localized at the membrane-cytoskeleton interfaces of cell to cell contacts, where they form a scaffold which is involved in the organisation of transmembrane proteins and other elements of signal transduction pathways (Bilder,

2001). This could perturb the control of the cell cycle by extracellular signalling pathways, which may contribute to the development of a neoplastic phenotype in E6 expressing cells (Glaunsinger et al., 2000).

In contrast, it was found that when an E6 mutant that was unable to bind to PDZ proteins was expressed at controlled levels during the virus lifecycle, the loss of the E6-PDZ protein interactions resulted in a reduction in the copy number at which the genome was maintained compared to wild-type (WT), but no change in the expression levels of PDZ proteins (Lee and Laimins, 2004). This appears on the surface to conflict with *in vitro* data which suggests that E6 induces the degradation of PDZ proteins, but it is possible that E6 is expressed at too low levels to be able to induce sufficient levels of degradation in raft culture. A reduction in the growth of cells harbouring the HPV E6 mutant genome compared to those harbouring the WT genome was also seen, although it is thought that this may just be a consequence of reduced genome copy number in the E6 mutant genomes compared to the WT genomes (Lee and Laimins, 2004).

Immune Evasion

The activation of the adaptive immune response to HPV infection is dependent on the presentation of viral antigens to antigen-presenting cells (APCs) resident in the skin (Tindle, 2002). The APCs that are present in the epithelium are called Langerhan cells (LC). The adhesion between LC and keratinocytes is mediated by E-cadherin (Tang et al., 1993). It has recently been shown that cell surface E-cadherin is reduced on HPV16-infected basal keratinocytes by 16E6 (Matthews et al., 2003). The mechanisms by which E6 reduces E-cadherin levels have not been elucidated, but it is thought that the purpose of this function of E6 in the virus life cycle is to actively evade the host immune response. It has been shown that the reduction in E-cadherin caused a proportional decrease in the numbers of LC in infected epithelium, in this way E6 expression infected basal cells may limit presentation of viral antigens by APCs to the immune system (Matthews et al., 2003). This function of E6 may also contribute to viral latency, and is almost certainly an important step in metastasis (Birchmeier and B ehrens, 1994). The E-cadherin-catenin complex plays a dominant role in the suppression of invasion and there is increasing evidence to support a causal

role of E-cadherin-catenin complex disruption in tumour invasion (Vermeulen et al., 1996).

1.13.2 The E7 protein

HPV16, E7 (16E7) is a dimeric phosphoprotein which has a zinc finger domain in its C-terminal region and the conserved motif LXCXE which is essential for binding to the under-phosphorylated form of the retinoblasma susceptibility protein (RB), in its N-terminal domain (Vousden, 1993; Stacey et al., 1994). E7 has sequence (outside the zinc-binding site) and functional similarity to proteins in other DNA tumour viruses such as adenovirus E1A and SV40 large tumour antigen (Barbosa et al., 1990; Dyson et al., 1992). The E7 protein of HPV16 is expressed in the basal and parabasal layers of the epithelium of productive lesions at low levels, and is upregulated in the intermediate layers of the epithelium during the late stages of the virus life cycle (Di Lonardo et al., 2001).

1.13.2.1 E7 functions

Replication

E7 is able to overcome negative growth signals in cells, including those mediated by transforming growth factor β, forcing post-mitotic cells in the parabasal and intermediate layers of the epithelium to enter S-phase of the cell cycle (Noya et al., 2001). The effects of E7 on the cell cycle are thought to be mediated by RB – dependent and independent pathways, the latter involves the direct targeting of cell cycle machinery. E7 proteins from both high- and low- risk HPVs associate with RB proteins, but generally the interaction between high-risk E7 proteins and RB are of higher affinity (Dyson et al., 1989; Munger et al., 1989) reviewed in (Vousden, 1995)).

RB proteins are pivotal in the control of three cellular pathways, namely terminal differentiation, cellular proliferation, and apoptosis. The RB family is considered an important negative regulator of the latter two processes, and as a key inducer of terminal differentiation (reviewed in (Weinberg, 1995)). Hypophosphorylated RB binds to the transactivation domain of members of the E2F transcription factor (TF)

family. This inhibits the ability of E2F to activate its E2F-responsive promoters. RB tethered to these promoter by E2F, can also actively inhibit the surrounding enhancer elements, further contributing to the repression of downstream S-phase genes in post-mitotic cells (Weintraub et al., 1992). Proliferation signals can prompt post-mitotic cells to re-enter the cell cycle, by inducing the hyperphosphorylation of RB. This in turn leads to the release of the E2F transactivation domain from the RB pocket domain, relieving RB-mediated repression of genes involved in DNA synthesis (Jones and Munger, 1996).

E7 binds to p130 and p107, which are members of the RB protein family (Dyson et al., 1992; Davies et al., 1993). These two proteins associate with two different groups of E2F TFs, which regulate the transcription of specific differentiation and proliferation genes, respectively. E7 causes the release of both groups of TFs, and in doing so inhibits differentiation, and promotes proliferation. Although important, the release of E2F from inhibitory complexes cannot alone relieve RB repression (Brehm et al., 1998).

RB proteins are in themselves potent repressors of E2F promoters, but the recruitment of mammalian histone deaceylase (HDAC) by RB to E2F complexes is also implicated in the silencing of E2F-responsive genes, by a process of nucleosome remodelling (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). Once bound to the promoter, HDACs removes acetyl groups from lysine residues present at the N-terminal tails of core histone proteins (reviewed in (Yoshida et al., 2003)). This unmasks the positive charge on the lysine residue, resulting in a tight interaction between the histone proteins and the DNA, resulting in the formation of heterochromatin and the repression of transcription. E7 combats HDAC gene silencing by inactivating RB and also by interacting with histone acetyltransferases and histone deacetylases (Brehm et al., 1999; Bernat et al., 2003).

E7 can increase the acetylation of histones specifically associated with E2F-mediated promoters that are active in E7 expressing cells this requires E7 binding to RB and E7

association with HDAC (Zhang et al., 2004). HPV16 E7 does not affect the steady state levels of HDACs, but E7 has been shown by some researchers to increase histone acetyl transferase (HAT) activity although others have reported no change (Huang and McCance, 2002; Avvakumov et al., 2003; Bernat et al., 2003; Zhang et al., 2004). Alternatively, to an increase in HAT activity it is possible that the interference with the function of HDAC by binding of E7 via Mi2β could block the accessibility of HDAC to its substrate (Brehm et al., 1999). Whatever the mechanism is it was found that the associations of 16E7 with HDAC and RB was required for the HPV genome to be maintained extra-chromosomally. Additionally, the interactions between 16E7 with HDAC and RB also appears to contribute to the immortalization ability of E7 expressing cells (Longworth and Laimins, 2004a).

Progression through the mammalian cell cycle is mediated by several cyclin-cdk complexes. The activities of these complexes are themselves regulated by cdk-activating kinases (CAKs), cdk-specific phosphatases, and cdk inhibitors (CKIs). Once initiated, the cell cycle is effectively autonomous. Initiation of the cell cycle occurs in response to mitogenic signalling, which increases the levels of D-type cyclins, which subsequently assemble with their catalytic partners, cdk 4 or cdk 6. The major substrate for these kinase complexes is RB. Late in G1 the D-type cyclins are down regulated, and cyclins E and A are produced. Cyclin E expression peaks at the G1/S transition, and cyclin A expression, which partially overlaps with that of cyclin E, accumulates in S-, and G2 phases of the cell cycle (Pines, 1999). The cyclin E-cdk 2 complex, like cyclin D-cdk 4/6 phosphorylates RB, but at additional sites, this forces cells to enter S-phase. The cyclin A-cdk 2 complex phosphorylates substrates required for the completion of S-phase, such as E2F (reviewed in (Vermeulen et al., 2003)).

E7 can increase the expression of the S-phase cyclins E and A and abrogate the functions of CKIs, $p21^{CIP1}$ and $p27^{KIP1}$ (Zerfass et al., 1995; Zerfass-Thome et al., 1996; Funk et al., 1997; Jones et al., 1997). The former is achieved by increased E2F-mediated transcription of cyclins E and A, and E7 has also been shown to be able to increase the stability cyclin E mRNA (Martin et al., 1998). E7 is also thought to

prevent the inhibition of cyclin-cdk activity by binding to the active sites of $p21^{CIP1}$ and $p27^{KIP1}$, which accumulate in E7 expressing cells (Zerfass-Thome et al., 1996; Funk et al., 1997; Jones et al., 1997).

E7 has been shown to associate directly with the S-phase cyclins which it upregulates the expression of i.e. cyclins E and A (Tommasino et al., 1993; McIntyre et al., 1996). These interactions bring E7 in close proximity to the cyclin binding partner cdk 2, enhancing the phosphorylation of substrates than are not normally targeted by cdk 2, such as Histone H1 (He et al., 2003). This suggests that E7 may play a role in the direct initiation of viral DNA synthesis as well as in the bypass of the G_1/S checkpoint in cells (Herrera et al., 1996; Xu et al., 1999). It is likely that this function of E7 contributes to the immortalization potential of the virus as well as chromosomal instability in E7 expressing cells, in part by inducing centrosome instability (He et al., 2003).

1.13.3 E1 protein

E1 is a phospho-protein which has two domains separated by a spacer sequence (Enemark et al., 2000). The N-terminal domain of E1 (E1N) is similar to that of the large T antigen of SV40, and to the EBNA1 protein of Epstein-Barr virus (EBV) (Clertant and Seif, 1984; Bochkarev et al., 1996). E1 like these other DNA tumour virus proteins, contains a nuclear localisation sequence (NLS), and a DNA-binding domain (DBD). In addition the C-terminal domain of E1 (E1C) has ATP-dependent DNA helicase activity (Yang et al., 1993). The E1 protein is thought to be expressed at very low levels *in vivo*, but has not yet been detected in PV lesions (Doorbar et al., 1990; Ozbun and Meyers, 1997; Ozbun and Meyers, 1998a).

1.13.3.1 E1 functions

The E1 ORF is one of the most highly conserved PV genes. For this reason the E1 proteins from different viruses are thought to have similar structures and functions, and studies so far generally support this assumption (Reviewed in (Myers and Sverdrup, 1997)). However, much of our understanding of E1 function comes from BPV1 studies.

Replication

E1-mediated replication has been shown to occur independently of the E2 protein and the HPV origin of replication (ori) when E1 is expressed at high levels (Seo et al., 1993b; Spalholz et al., 1993; Kuo et al., 1994; Bonne-Andrea et al., 1995; Santucci et al., 1995). In these experiments and others, E1 was found to form hexameric complexes at multiple sites on plasmid DNA, which unwound the DNA and initiated genome replication (Hughes and Romanos, 1993; Yang et al., 1993). This demonstrates that E1 plays a central role in genome replication. However, *in vivo* efficient replication is likely to initiate at the ori, and involve the E2 protein in addition to the E1 protein (Ustav and Stenlund, 1991; Ustav et al., 1991; Seo et al., 1993a). This is because physiologically, E1 is expressed only at very low levels, and only associates with DNA non-specifically in the absence of E2 (Dixon et al., 2000). Together this means that the probability of E1 di-hexamers being formed on the genome spontaneously is low. However, when E1 and E2 proteins are co-expressed, E1 is targeted to the origin of HPV replication (ori) where it binds cooperatively with E2 to the E1 binding region found next to the E2 BS#3 (Mohr et al., 1990; Blitz and Laimins, 1991; Yang et al., 1991; Le Moal et al., 1994).

An association between the E1 N-terminal ATP-binding domain, and the E2, transactivation domain (E2TAD), is thought to be essential for the cooperative binding of an E1-E2 complex to the ori (Sarafi and McBride, 1995; Sedman and Stenlund, 1995; Berg and Stenlund, 1997; Moscufo et al., 1999). In addition, a comparatively weaker association between the DNA-binding domains (DBDs) of both E1 and E2, is also thought to contribute to cooperative binding (Benson and Howley, 1995; Woytek et al., 2001). The formation of an E1-E2-ori complex causes distortion of the DNA at the ori, which facilitates the step-wise recruitment of additional E1 molecules, to the region of the ori that is recognised by E1 (Gillette et al., 1994; Sanders and Stenlund, 1998). This region contains 6 overlapping hexameric repeats (AACNAT, with N being any base except G), which vary in their binding affinities for E1 dimers. Chen *et al* has proposed a model for the assembly of the initiator complex at the PV ori, which has been summarised in Figure 1.4 (Chen and Stenlund, 2001). Following the recruitment of additional E1 molecules to the ori, it is essential that E2 is released to enable E1 to be assembled into an active complex (Lusky et al., 1994; Titolo et al.,

1999). E2 is displaced from the initial targeting complex by the competitive binding of ATP to E1 (Sanders and Stenlund, 1998).

Although the viral E1 and E2 proteins are essential for the formation of the replication complex at the ori, cellular proteins are also thought to contribute to the efficiency of formation of this complex. For example, heat shock proteins, hsp40 and hsp70, can interact with E1 and can enhance E1 binding to the ori, and are thought to favour the formation of di-hexamers on the ori, as opposed to hexamers (Liu et al., 1998; Lin et al., 2002). The former is essential for the bi-directional replication mechanisms employed by PVs during the productive stage of the PV life cycle (Flores and Lambert, 1997). The local arrangement of histones at the ori is also likely to be important for the initiation of viral replication. E1 can associate with histone H1, and Ini1/hSNF5, the latter is a component of the SWI/SNF complex which has HAT activity (Swindle and Engler, 1998; Lee et al., 1999). These interactions may be important for the remodelling of the HPV genome at the ori.

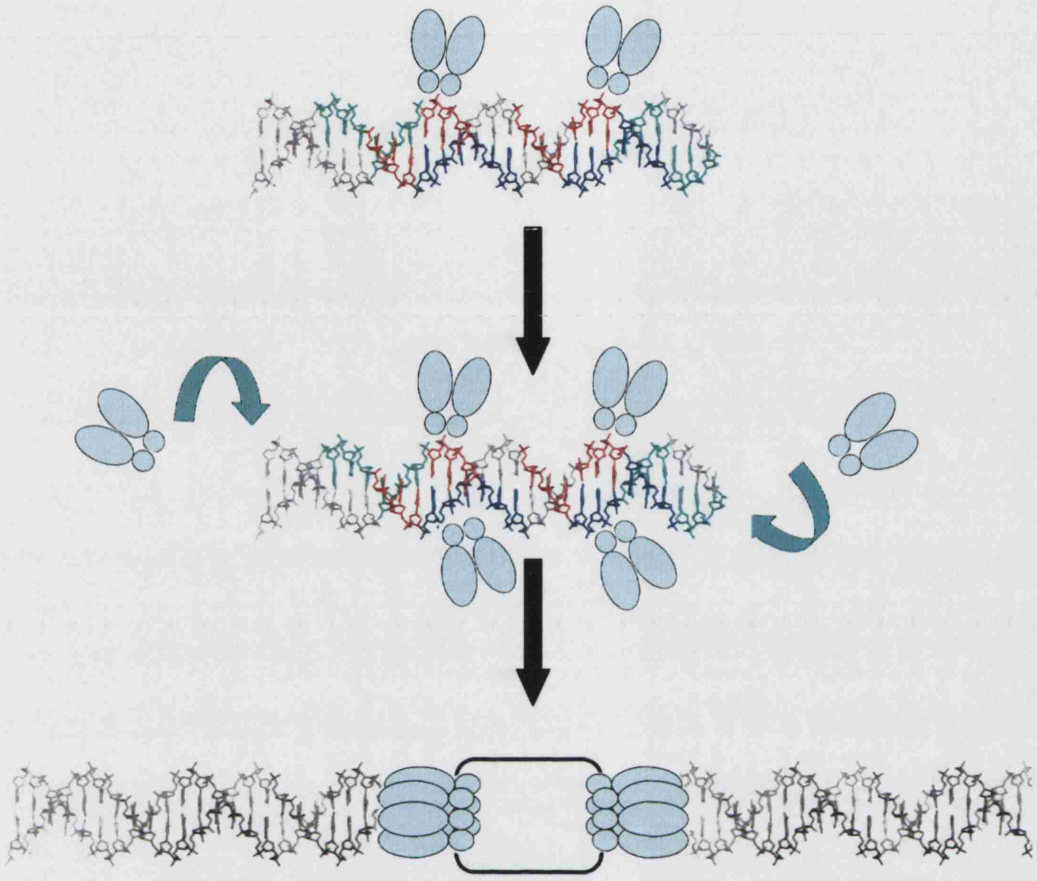


Figure 1.4 Model for E1 initiator complex assembly
Two E1 dimers (N-terminal domain is represented by a blue circle and C-terminal domain by a blue oval) are targeted to E1 binding sites (BSs) 2 and 4 (coloured red on the diagram of the BPV E1 binding region) by the E2 protein (not shown). The E1 dimers bound to sites 2 and 4 facilitate binding of two more E1 dimers to the comparatively weaker E1 BSs, 1 and 3 (coloured blue). The E1 BSs 1-4 have overlapping recognition sequences but can still be occupied by four E1 dimers simultaneously without steric hindrance. This is because overlapping sites are exposed on different faces of the DNA helix. It has been predicted that an additional two E1 dimers bind to the putative E1 BSs 5 and 6 (coloured green) but this has not been shown. Occupation of E1 BSs 5 and 6 is thought to facilitate the formation of two E1 hexameric helicase that can unwind DNA enabling bi-directional DNA replication to proceed (Adapted from (Chen and Stenlund, 2001).

Transcription

E1 has been shown to modulate the ability of E2 to transactivate and transrepress the viral early promoter in a concentration-dependent manner in transient assays (Sandler et al., 1993; Le Moal et al., 1994; Ferran and McBride, 1998). The former involves cooperative binding of an E1-E2 complex to the ori. It has been suggested that the association between E1 and E2 proteins may stabilise the transcriptionally active conformation of the E2 transactivation domain (TAD), allowing E1 to increase transcription from the early promoter at low protein concentrations (Piccini et al., 1995; Parker et al., 2000). E1 and E2 associate at three different sites. Two of these are also important for the cooperative binding of E1 and E2 to DNA. The third association that occurs between the E1 DBD and the E2 TAD, is also thought to contribute to the stabilisation of E2 TAD (Piccini et al., 1995; Woytek et al., 2001). The HPV18, E1 protein has also been shown to have intrinsic transactivational ability when it is efficiently targeted to E2 BSs located upstream of a heterologous promoter (Demeret et al., 1998). This property of E1 is possibly related to its ability to concentrate cellular TFs, such as the SWI/SNF complex within the vicinity of a promoter (Lee et al., 1999).

E1 repression of early transcription has also been shown to occur when E1 is expressed at high levels *in vitro* (Le Moal et al., 1994). The over-expression of E1 may sequester E2 and other cellular TFs away from their active sites, this affect is known as TF squelching, and may explain E1 repression in experimental systems. However, *in vivo* E1 is thought to be expressed at very low levels, which is thought to be due to the presence of a strong splice donor site near to the start of the E1 ORF.

1.13.4 The E2 protein

HPV16, E2 is composed of two domains, the N-terminal transactivation domain (amino acids 1-220) and the C-terminal dimerisation/DNA binding domain (DBD, amino acids 260-365), separated by a flexible hinge region (Giri and Yaniv, 1988; Gauthier et al., 1991; Sakai et al., 1996). A NLS is thought to be present in the C-terminal domain of the protein (Sanders et al., 1995; Bellanger et al., 2001). HPV16 E2 (16E2) is thought to be expressed at low levels in the nuclei of cells in the basal

layer of the epithelium, and at high levels mostly in the nucleus and also in the cytoplasm of cells that are actively replicating the HPV genome (Maitland et al., 1998; Ozbun and Meyers, 1998a; Stevenson et al., 2000).

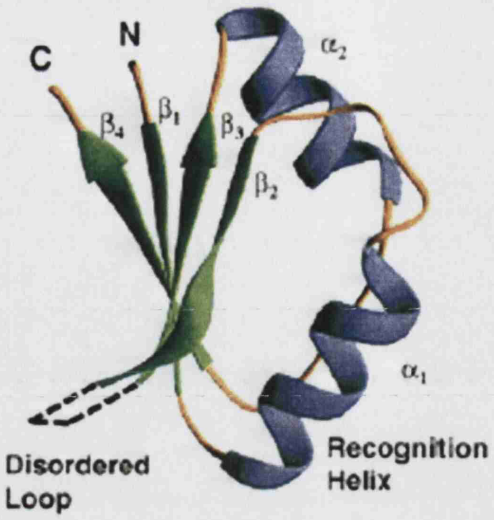
A putative E2 repressor protein is also encoded by the HPV16 ORF and is called E2C (Doorbar et al., 1990). Transcripts encoding E2C, and other truncated E2 repressor proteins, have been identified in several PV types (Lambert et al., 1987; Liu et al., 1995; Stubenrauch et al., 2001). E2C is an N-terminally truncated form of E2, which is thought to contain the hinge and the C-terminal domain of the full-length protein. E2C in principle is able to form homo- and heterodimers with full-length E2, which can repress transcription from the early promoter, by competing with full-length E2 homodimers for binding to E2-specific sites on DNA (Romanczuk et al., 1991).

Structure of E2-DNA complexes

The consensus sequence of the E2 binding site (BS) is ACCg $(N)_4$ cGGT (bases in lower case are the preferred nucleotides, and $(N)_4$ represents a spacer between the conserved palindromic half sites) (Figure 1.5a). The spacer sequence affects the affinity that 16E2 has for its BS, even though it does not make contact with E2. This is because DNA bending of the hinge region by E2 is necessary for symmetrical contact to be made between E2 and the palindromic half sites of the DNA (Sanders and Maitland, 1994; Hou et al., 2002). HPV16 E2 displays increased affinities for consensus binding sites which have relatively rigid spacers (A/T rich) (Nelson et al., 1987). The introduction of flexibility in the spacer DNA of the E2 binding site, whether conferred by sequence or nicks can drastically reduce the affinity 16E2 has for its binding site, because the energy of 16E2-DNA contacts is not sufficient to counter inherent flexibility in the DNA (Hines et al., 1998).

The C-terminal domain of E2 has two surface α-helices which become inserted into the major grooves of the DNA, without affecting the dimerisation interface (four-stranded anti-parallel β-sheet) of E2. The β2 and -3 strands of the dimerisation surface of E2 are connected by a disordered loop (Figure 1.5b). This region of BPV1, E2 undergoes structural changes upon DNA binding, as the loop assumes an ordered

a



b

BS 1: ACCG AAAC CGGT

BS 2: ACCG AAAT CGGT

BS 3: ACCG TTTT GGGT

BS 4: ACCG AATT CGGT

c

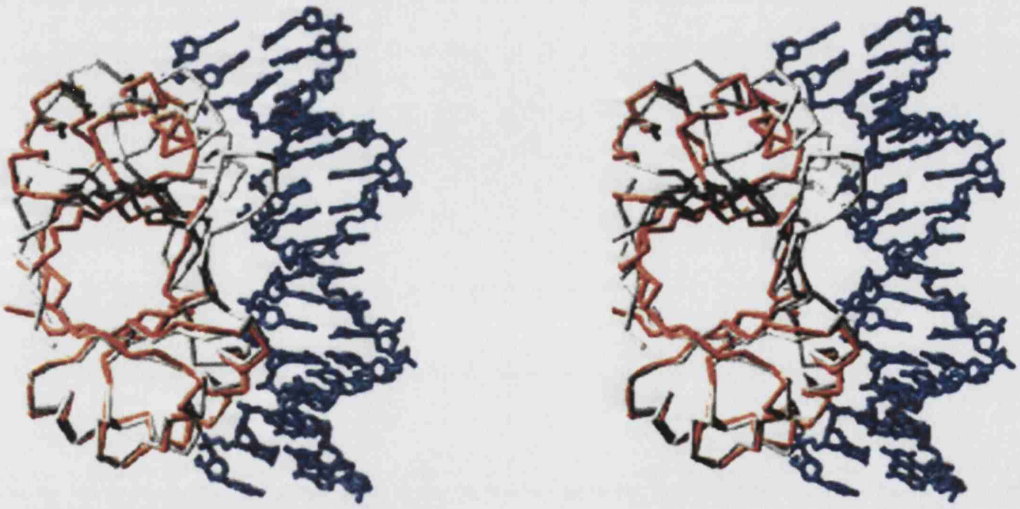


Figure 1.5 The E2 protein and DNA

(a) Polypeptide fold of a monomer of the HPV16 E2 DNA-binding domain indicating the four-stranded anti-parallel β-sheet (green) and the two surface α-helices (blue) (Image was taken from Hegde and Androphy, 1998). (b) The sequences of the 4 E2 BSs in the HPV16 LCR are shown. Note that BS 3 does not conform exactly to the 16E2 consensus sequence, ACCg $(N)_4$ cGGT (bases in lower case are preferred nucleotides and $(N)_4$ forms a spacer between the conserved palindromic half sites) or with the preferred spacer sequence AA(A/T)N (Sanders and Maitland, 1994). (c) Stereo view of a superimposition of the structures of the free HPV16 E2 DNA-binding domain (red) and the BPV1 E2 DNA-binding domain bound to DNA (gray). One subunit (lower) of each protein was superimposed. The DNA conformation found in the BPV1 E2-DNA complex is shown in blue (Image was taken from Hegde and Androphy, 1998).

conformation such a disorder to order transition is not seen when 18E2 binds to DNA, but is thought to occur when 16E2 associates with its BS (Figure 1.5c).

1.13.4.1 Functions of E2

Replication

The primary role of E2 in replication is to recruit the viral DNA helicase, E1, to the origin of HPV replication (ori). The two proteins then cooperatively bind to the core ori sequence, which is composed of the E1-binding region and the neighbouring E2BS #3. The HPV ori contains another two E2 BSs (BS #1 and BS #2), which contribute to efficient HPV replication. These sites, when occupied, act synergistically with the E1-E2-ori complex, by bending the DNA at the ori in a way that encourages E1 oligomerisation (Sverdrup and Khan, 1994; Sverdrup and Khan, 1995; Gillitzer et al., 2000; Chen and Stenlund, 2001). E2 like E1, has been shown to associate with several cellular proteins, some of these have been shown to be important for the initiation and progression of PV replication (Demeret et al., 1995; Melendy et al., 1995; Masterson et al., 1998).

Genome maintenance

E2 plays a direct role in the mechanism of HPV replication, and in doing so affects genome maintenance, apart from this E2 is also involved in the partitioning of the genome between daughter cells in the basal layer of the epithelium. Evidence for this comes from the observations that in mitotic cells E2 can co-localise with mitotic spindle fibres (Ilves et al., 1999; Bastien and McBride, 2000). More recently it has been shown that HPV E2 can associate with α-, β- and γ- tubulins which are the three major proteins that form mitotic spindles. Both the N- and C- terminal domains of E2 are thought to mediate the interactions of E2 with mitotic spindles (Van Tine et al., 2004). HPV16 E2 also associates with bromodomain containing 4 (Brd4). An interaction between BPV1 E2 and Brd4 has also been identified; this interaction has been shown to be important for genome segregation and ultimately for the persistence of viral genomes in a PV infection (You et al., 2004). Thus it is possible that the interaction between HPV16 E2 and Brd4 may also be important for this mechanism.

The C-terminal domain of E2 can also make contacts with E2 BSs in the HPV genome (Androphy et al., 1987; Giri and Yaniv, 1988). Together the interactions of E2 with mitotic spindles and the HPV genome are likely to allow viral genomes to be segregated between daughter cells.

Apoptosis induction

The expression of E2 proteins from high-risk HPVs, in HPV-immortalised cell lines has been shown to induce apoptosis (Sanchez-Perez et al., 1997; Desaintes et al., 1999). SiHa cells contain 1-2 copies of the HPV16 genome integrated into the host chromosome (Callahan et al., 1992). When low levels of 16E2 were ectopically expressed in SiHa cells, an increase in transcription from the HPV16 early promoter was seen (Sanchez- Perez et al., 1997). This in turn leads to an accumulation of E7 protein, as the major transcripts produced from this promoter encodes E6*I and E7 (Nilsson et al., 1996; Sanchez-Perez et al., 1997). This creates an imbalance in E6 and E7 expression, and ultimately can induce apoptosis in a p53-dependent manner (Sanchez-Perez et al., 1997).

High levels of expression 18E2 protein has been shown to cause apoptosis independently of E6 and E7 and is thought to involve the N-terminal domain of E2 and cleavage of the initiator of the extrinsic apoptosis pathway, caspase 8 (Frattini et al., 1997; Desaintes et al., 1999; Webster et al., 2000; Demeret et al., 2003). In contrast 16E2 has only been shown to cause apoptosis in a p53-dependent manner, although like 18E2 this mechanism is independent of E6 and E7 viral oncogene expression (Webster et al., 2000). Thus, it is possible that the mechanisms that the E2 proteins from HPV16 and 18 induce apoptosis may be different. However, the putative role of E2-mediated apoptosis in virion release may be conserved between these high-risk HPVs (Demeret et al., 2003).

Transcription

HPV early gene transcription is activated by the interaction of host cell transcription factors (TFs) with the LCR. E2 can further modulate the activity of this promoter in a concentration-dependent manner (Sanders and Maitland, 1994; Stubenrauch and Pfister, 1994; Stubenrauch et al., 1996; Steger and Corbach, 1997). E2 is a *trans*-

acting TF, because it can associate with DNA and a collection of transcription regulatory proteins. These may induce conformational changes in E2, or may affect the accessibility or affinity of E2 for DNA. Occupancy of E2 BSs is the principal mode by which E2 regulates the early promoter. However, the methylation state of the chromatin, which is thought to change during the differentiation-dependent virus life cycle, may also be important in the context of a HPV infection.

The LCR of high-risk HPVs have 4 E2 BSs (BS 1-4). BSs 1-3 are located in the ori, and are involved in DNA replication as well as transcription. BS 3 is juxtaposed to the E1 binding region, and is essential for E1-E2 cooperative binding to DNA. BS 1 and 2 are separated from each other by 2-4 bp and are sometimes called the promoter proximal E2 BSs (Figure 1.3). BS 1 overlaps with *cis*-components which are required for the binding of basal transcription factors (TFs), such as that for the TATA binding protein (TBP) and TFIID, and BS 2 is located in close proximity to the Sp1 TF, BS. Occupancy of the promoter proximal E2 BSs is thought to result in the displacement of these key general TF (Romanczuk et al., 1990; Dostatni et al., 1991; Thierry and Howley, 1991; Dong et al., 1994; Tan et al., 1994; Stubenrauch et al., 1996; Demeret et al., 1997). BS 4 or the distal binding site promotes transactivation when occupied (Steger and Corbach, 1997).

The C-terminal domain of 16E2 has been shown to have similar binding affinities for all four HPV16, E2 BSs, despite differences in their DNA sequences (Sanders and Maitland, 1994). This is in contrast to the absolute affinity values (off rates), which vary 100 fold (Sanders and Maitland, 1994). Absolute affinities are a measure of the stability of the E2-DNA complexes. The most stable complex formed was between E2 and BS 4, and the least stable complex was between E2 and the degenerative BS 3. It has also been shown that the stability of the E2 complexes with BS 1 and BS 2, were similar and within the range of the E2-BS 4 complex (Sanders and Maitland, 1994). Similar binding constants were also found using the full length E2 protein, suggesting that the C-terminal domain of E2 is the sole determinant of DNA binding affinity (Thain et al., 1997).

This means hypothetically that at low concentrations of E2, BS 4 is likely to become occupied before the other E2 BSs. E2 bound at the distal BS is thought to activate transcription by interacting with Sp1, and possibly other cellular TFs bound close to the promoter, by participating in the formation of a stable DNA loop (Li et al., 1991). When E2 levels increase to a critical point, TFs such as Sp1 and TBP will be displaced from promoter proximal sites by E2, ultimately reducing but not abolishing transcription (Steger and Corbach, 1997).

The role of BS 3 in transcriptional regulation is not well characterised. HPV18, E2 has been shown to repress transcription by associating with this site with relatively high affinity (Steger and Corbach, 1997). In contrast, BPV1 and HPV16, E2, form only weak complexes with their respective BS 3 (Li et al., 1989; Sanders and Maitland, 1994; Steger and Corbach, 1997). However, studies which assess the effect of E1-E2, upon the E2 BS 3 in the context of the ori, suggest that occupancy of this site by the E1-E2 complex, massively activates transcription (Le Moal et al., 1994; Piccini et al., 1995; Demeret et al., 1998). E2 BSs become saturated at very low protein concentrations, making it difficult to study the full range of effects of E2 on the native HPV LCR in cell-based transcription assays. In these systems E2 is usually constitutively expressed at high levels from mammalian expression vectors, and so the experiment itself is commonly biased towards the analysis of E2-mediated repression (Sanders and Maitland, 1994).

TFs such as the breast cancer susceptibility gene 1 (BRCA1) and CBP/p300 are recruited to the LCR in an E2-dependent manner i.e. are tethered to the E2 BSs via E2 protein (Bouallaga et al., 2003; Kim et al., 2003a). These factors have been shown to effect E2-mediated transcription. BRCA1 recruits chromatin remodelling enzymes to the LCR and CBP/p300 is itself a histone acetyltransferases (HAT). In addition BRCA1 and CBP/p300, like E2, associate with different components of the basal transcription machinery, as does the E2 associated DNA binding protein toperisomerase II β-BP1 (Scully et al., 1997; Blobel, 2000; Bakshi et al., 2001; Boner et al., 2002). In combination with each other, some of these cellular factors and also others are specifically involved in the formation of a higher-order nucleoprotein

complex (enhanceosome) which is thought to be required for high levels of transcription.

In undifferentiated cells HPV DNA is associated with nucleosomes. The position of three nucleosomes in the LCR have been mapped, and cover the middle of the enhancer sequence, the ori, and also part of the E6/E7 ORFs (Stunkel and Bernard, 1999). Examination of the methylation patterns of the HPV16 LCR in undifferentiated cells revealed that nucleosomally organised DNA segments are frequently hypermethylated (at CpG motifs), whereas linker DNA segments are usually hypomethylated (Kalantari et al., 2004). It is thought that promoter activation is likely to be reduced but not completely absent in the basal cells of the epithelium, as the tight association of methylated DNA segments with nucleosomes may prevent the binding of a lot of TFs to the LCR (Harrington et al., 1988; Bechtold et al., 2003). However, as cells differentiate, the methylation patterns of HPV DNA are not maintained (Kim et al., 2003b; Etoh et al., 2004). This may initiate nucleosome displacement from the LCR and downstream promoter which would favour viral gene transcription.

1.13.5 The role of E2-mediated transcription in the virus life cycle

The ability of E2 to both activate and repress early gene transcription has been shown in transient reporter assays. On the basis of these studies, and the observation that the E2 ORF is frequently lost during malignant development, it was proposed that E2 may be essential for the regulation of viral early gene expression in the context of the PV life cycle. However, recent reports have begun to question the *in vivo* significance of E2 transcriptional activity.

Stubenrauch *et al* analysed the role of two characterised N-terminal E2 mutants in the HPV31 life cycle (Sakai et al., 1996; Stubenrauch et al., 1998). The mutants analysed were R37A and A73I and are located on the N-terminal dimerisation interface of E2 (Antson et al., 2000). These mutants in HPV16 and 31 E2 proteins were shown to have no transactivation ability compared to the wild-type (WT) protein on an artificial E2-responsive promoter, but their effects on the WT LCR were comparable to WT E2

i.e. no significant levels of stimulation of the downstream promoter was seen at low levels of E2, but at high levels both mutants and WT E2 were found to repress transcription (Sakai et al., 1996; Stubenrauch et al., 1998). It was also shown that both mutants were able to replicate an ori-containing plasmid in cell-based assays, but the R37A mutant did display partially reduced replication capacity compared to the WT E2 protein (Sakai et al., 1996; Stubenrauch et al., 1998). This is despite the ability of R37A being able to bind to E1 at levels that were comparable to WT E2 (Sakai et al., 1996). HPV31 genomes harbouring either R37A or I73A E2 mutations were examined in the context of the raft cultures and compared to the WT genome (Stubenrauch et al., 1998).

It was found that both mutant genomes had reduced levels of genome amplification relative to WT, but only the difference between WT and the R37A harbouring mutant was considered to be significant. This mutant also showed reduced early gene transcription compared to WT and failed to show any late gene expression in raft cultures. The I73A mutant did not display any difference in transcription from the early promoter relative to WT, but did cause a reduction in transcription from the late promoter. The authors conclude based on these observations that while the transactivation ability of E2 is not essential for induction of early or late gene transcripts, but may augment them.

This conclusion seems to be an over interpretation of the data, as it was not found that the E2 mutant proteins behaved differently from WT E2, when the effect of these proteins on the WT LCR was assessed. In addition, both E2 mutants that were used in life cycle studies were found to bind to E1 and to DNA at levels that were comparable to that of WT E2 (Sakai et al., 1996). Despite this, this study and the one by Stubenrauch *et al* neglected to assess the contribution of E1/E2-mediated transactivation on the virus life cycle, which is presumably unaffected by the N-terminal E2 mutations (Sakai et al., 1996; Stubenrauch et al., 1998). This assumption is made based on the observation that the mutant E2 proteins were able to function in ori-dependent replication, a process which also involves E1 and E2 interaction and cooperative binding of the complex to the ori and coincides with late gene transcription (Burnett et al., 1990). However, an interesting observation from this

study was that the E2 mutants that incidentally fall on the N-terminal dimerisation interface of E2 were able to reduce or abolish transcription from the late promoter. This raises the possability that E2 may possibly be able to regulate transcription from the late HPV promoter as well as the early promoter, although it cannot be ruled out that the reduction in genome copy number observed in cell lines that harboured the mutant genomes in this study may account for the observed reduction in late transcription.

Another study by Bechtold *et al* entitled "Human papillomavirus type 16 E2 protein has no effect on transcription from episomal viral DNA", concludes that the role of E2 as a TF is secondary to that of replication in the virus life cycle (Bechtold et al., 2003). In this study the effect of exogenously expressed E2, on the HPV16 early promoter in the context of the HPV genome was examined. It was found that the conformation of the chromatin was important for E2-mediated transactivation, and more specifically the acetylation status of the histones that were present at the p97 promoter. In undifferentiated cells it was found that the HPV16 promoter in its natural episomal context was unaffected by 16E2 expression in cells. These findings can be correlated to the early stage of the HPV life cycle, and fortify our understanding of the modulation of HPV transcription by E2, but these findings should not be extrapolated to the late stages of the virus life cycle. This is because the chromatin structure and the expression of both viral and cellular modulators of the LCR changes with differentiation (Apt et al., 1996; Ozbun and Meyers, 1998a; Kim et al., 2003b). This is supported by the finding that the methylation status of the HPV DNA changes with differentiation; this is likely to influence the effect of E2 on transcription from the LCR (Kalantari et al., 2004).

In summary, these studies demonstrate that E2 is not an important transcriptional regulator in the early stage of the HPV life cycle, but these papers do not fairly test the effect of E1/E2-mediated transcription on viral transcription in the late stages of the HPV life cycle.

1.13.6 The E5 Protein

HPV16 E5 is a strongly hydrophobic membrane protein. E5 has been shown to associate primarily with the endoplasmic reticulum (ER), but also can associate with the Golgi apparatus, the nuclear membrane, and the plasma membrane (Burkhardt et al., 1989; Conrad et al., 1993). A hypothetical structure for E5, predicts that the protein is composed of three membrane helices, and short N- and C-termini, that are thought to extend beyond the lipid bilayer (Ullman et al., 1994).

1.13.6.1 E5 functions

In BPV, E5 is the major transforming protein. In contrast, HPV16 E5 (16E5) is only possess weak transformation ability compared to 16E6 and E7, although E5 does compliment E7-mediated cellular transformation (DiMaio et al., 1986; Leptak et al., 1991; Valle and Banks, 1995). The E5 ORF is thought to be transcribed on all the polycistronic mRNAs produced by the major early and late promoters, with the exception of transcripts encoding the viral capsid proteins (Baker and Calef, 1996). This suggests that E5 may play a role in both the non-productive and productive stages of the life cycle (Fehrmann et al., 2003; Genther et al., 2003).

Immune evasion

HPV proteins are in general expressed at low levels in the basal layer of the epithelium. This helps the virus, to passively avoid detection by the immune system. In addition, the E5 protein is thought to directly combat cytotoxic T lymphocyte (CTL)-mediated clearance of virus-infected cells, by reducing the surface expression of the major histocompatibility class I complexes, HLA-A and HLA-B, which present viral peptides to CTLs (Ashrafi et al., 2005). This is achieved by the retention of MHC complexes in the Golgi apparatus, and the deregulation of the levels of proteins that are involved in the maturation and transport of MHC class I complexes to the plasma membrane by the E5 protein (Leykauf et al., 2004).

Apoptosis prevention

The expression of E7, and E2, in the lower layers of the epithelium can promote apoptosis. This cell fate can be counteracted by E6 and E5 proteins. Epithelial raft

cultures which express HPV16 E5, are protected from FasL- (Fas ligand) and TRAIL- (tumour necrosis factor-related apoptosis-inducing ligand), mediated apoptosis (Kabsch and Alonso, 2002; Kabsch et al., 2004). The mechanism by which E5 protects against apoptosis has not been deciphered, although inhibition of death receptor signalling is suspected. The importance of the anti-apoptotic function of E5 in the context of the virus life cycle is however questionable, especially in the light of recent studies in which this function of E5 was found to be dispensable for the HPV life cycle (Fehrmann et al., 2003; Genther et al., 2003).

Viral genome replication

E5 is thought to have a quantitative role, in the genome amplification stage of the HPV life cycle. In two parallel studies, mutant HPV genomes which do not express E5 proteins, were found to cause a 2-fold decrease (relative to WT genomes), in the number of cells that were actively supporting vegetative HPV replication (Fehrmann et al., 2003; Genther et al., 2003). This effect of E5 has been linked to receptor signalling pathways, and in particular to epidermal growth factor receptor (EGFR) signalling (Pim et al., 1992; Straight et al., 1993; Gu and Matlashewski, 1995; Crusius et al., 1998; Tomakidi et al., 2000).

E5 stimulates this latter pathway in a ligand-dependent manner, via its association with the vacuolar proton ATPase protein (v-ATPase) (Hwang et al., 1995). This interaction is thought to cause the disruption of the endosomal membrane pore, of which v-ATPase is a component, delaying the process of endosome acidification (Straight et al., 1995). This increases EGFR recycling, between endosomes and the cell membrane, which increases the number of EGFR on the cell surface, thus amplifying EGFR signalling in the presence of hormone. E5 also associates with the EGFR directly, which enables EGFR signalling to also occur in the absence of ligand stimulation (Conrad et al., 1993). This enables E5 to increase mitogenic signalling and delay differentiation, ultimately increasing the propensity of E5-expressing cells to proliferate. In this way E5 may contribute to the productive stage of the life cycle.

1.13.7 The capsid proteins

PVs encode two viral structural proteins, the major capsid protein, L1, and the minor capsid protein, L2. These proteins are expressed from the differentiation-dependent promoter, in the upper layers of infected tissue after viral genome amplification (Ozbun and Meyers, 1998b). Both proteins have C-terminal NLSs, consisting of clusters of basic amino acids (Zhou et al., 1991). These are recognised by adapter, and import, receptors of the karyopherin (Kap) family, which are responsible for the shuttling of L1 and L2 between the nucleus and cytoplasm (Nelson et al., 2002; Darshan et al., 2004).

1.13.7.1 Function of the capsid proteins

L2 is synthesized prior to L1, and accumulates at nuclear structures called nuclear domains 10 (ND10s). ND10s are the sites at which viral genome replication, and packaging, is thought to occur (Doorbar et al., 1997; Swindle et al., 1999; Florin et al., 2002). L1 pentamers, which assemble in the cytoplasm, are recruited to ND10s by L2, following the L2-induced release of Sp100, which is a major ND10-associated protein (Okun et al., 2001; Florin et al., 2002). A single L2 molecule interacts with each L1 pentamer, via sequences in its N- and C-terminal domains. The N-terminal interaction is comparatively weaker, than that of the C-terminal, but both are required for encapsidation (Okun et al., 2001). L2 has also been shown to be involved in the translocation of E2 to ND10s (Day et al., 1998). Since E2 binds to the viral genome at multiple sites, the recruitment of E2 to ND10s is thought to facilitate the selective packaging of viral DNA into virions.

1.13.8 The E1^E4 protein

The E1^E4 protein was first discoverd in 1986 by Doorbar *et al*, following the examination of HPV1 lesions using antisera raised against HPV1 gene fragments expressed in *E.coli* (Doorbar et al., 1986). The E1^E4 protein is encoded by a spliced mRNA and derives its first few amino acids (five amino acids in the case of 16E1^E4) from the E1 ORF, whilst the remainder come from the E4 ORF (Nasseri et al., 1987; Doorbar et al., 1988; Doorbar et al., 1990). The E4 ORF lies within that of E2,

although in a different reading frame (Figure 1.2). The E1^E4 protein is encoded by the early and late HPV promoter, but in transcripts from the early promoter E1^E4 is the third ORF whereas in the latter it is the first, thus it is thought that E1^E4 is only likely to be efficiently translated from late transcripts (Stacey et al., 1995). Thus the high levels of E1^E4 expression observed late during infection results from increased transcription from the differentiation-dependent (late) promoter (Hummel et al., 1992).

E1^E4 proteins are usually small (16E1^E4 is 10K) and tend to be unusually rich in proline and charged residues, and many have conserved leucine motifs (reviewed in (Doorbar and Myers, 1996)). In addition the C-terminal domain of E1^E4 proteins are found to be conserved within cutaneous and within mucosal HPV types, because of this it has been proposed that E1^E4 may play a tissue-specific role during infection (Smith and Campo, 1985). Although there are exceptions, namely the E1^E4 proteins from HPV1 and HPV2 are not well conserved in the context of the cutaneous group of viruses (Doorbar et al., 1989). The C-terminal domain of E1^E4 has been shown to self-associate forming multimeric complexes (Brown et al., 1988; Doorbar et al., 1996; Ashmole, 1998). HPV16 E1^E4 has specifically been shown to form dimers, hexamers, but may also form trimers and tetramers (Roberts et al., 1997; Wang et al., 2004). Mutation of one of the conserved cysteine residues in the C-terminal domain of 16E1^E4 prevents multimerisation, suggesting that disulfide bond formation may stabilise 16E1^E4 oligomerisation (Roberts et al., 1997). However, as the mutation of a single cysteine residue does not prevent E1^E4 self-association, disulphide bonding alone is not enough for E1^E4 multimerisation (Roberts et al., 1997; Ashmole, 1998).

E1^E4 is typically expressed to high levels in productively infected lesions, in fact HPV1 E1^E4 protein can constitute up to 30% of the total protein of a wart (Doorbar et al., 1989). HPV16 E1^E4 is also abundantly expressed, but predominantly in the cytoplasm of cells in the intermediate and superficial layers of the epithelium (Doorbar et al., 1997; Middleton, 2003). The appearance of E1^E4 protein in a PV infection coincides with the onset of viral genome amplification (Breitburd et al., 1987; Doorbar et al., 1997; Peh, 2002).

In addition to the different multimeric forms of E1^E4, phosphorylated and N-terminal cleaved forms have also been described (Breitburd et al., 1987; Doorbar et al., 1997; Knight et al., 2004). Several N-terminally truncated E1^E4 species i.e. E1^E4 16K, 11kDa and 10K in size as well as full-length E1^E4 (17K) have been described in HPV1 lesions (Breitburd et al., 1987; Doorbar et al., 1988). The truncated forms of 1 E1^E4 are more abundant in the upper layers of differentiated epithelium whereas full-length E1^E4 and the 16K species are more abundant in the lower layers, suggesting that the proteolytic processing of E1^E4 may be differentiation-dependent (Breitburd et al., 1987). Recently, different functions for the 17K and 16K forms of 1E1^E4 have been described, suggesting that proteolytic cleavage of E1^E4 may be a method of regulating the function of E1^E4 in the virus life cycle (Knight et al., 2004).

For HPV16 E1^E4 an N-terminally truncated form has also been described (Doorbar et al., 1997). This form of E1^E4 is co-expressed with the full length protein but gives rise to a slightly different intracellular staining pattern. Full length 16E1^E4 has a filamentous pattern in the cytoplasm of most cells *in vivo* due to its association with the keratin intermediate filament network, but in some cells E1^E4 is found in perinuclear bundles which are produced by the E1^E4 mediated collapse of the cytokeratins network (Doorbar et al., 1997). An antibody which specifically recognises the extreme N-terminus of E1^E4 stains the periphery of the collapsed E1^E4/keratin bundles whereas an antibody which recognises both truncated and full-length forms of E1^E4 stained the centre of the bundle comparatively more strongly (Doorbar et al., 1997).

HPV11 E1^E4 has been shown to be phosphorylated by protein kinase A (PKA) and by mitogen activated protein kinase (MAPK) at two consensus sites in the central portion of the protein (Bryan and Brown, 2000). In addition HPV1 E1^E4 has also been shown to be phosphoylated by PKA *in vitro* (Grand et al., 1989). The computational analysis of HPV16 E1^E4 has identified several consensus phosphorylation sites on the protein (Appendix I). These include multiple MAPK consensus sites and a PKA site. Although the position of 16E1^E4 MAPK phosphorylation site(s) have not been mapped, 16E1^E4 has been shown to be

phosphorylated *in vitro* by MAPK (Bryan and Brown, 2000). It has also been shown for HPV1 E1^E4 that the extent of E1^E4 phosphorylation increases with epithelial differentiation, suggesting that the phosphorylation of E1^E4 may effect its function (Breitburd et al., 1987).

1.13.8.1 Functions of E1^E4

Replication

The coincidence of genome amplification and high levels of E1^E4 expression, was first noted in a HPV1 infection by Breitburd *et al* (Breitburd et al., 1987). Based on this observation, it was postulated that E1^E4 proteins may play a direct role in viral genome replication. More recently, it has been shown that CRPV E1^E4, is essential for genome amplification and late viral gene expression in viral infections in rabbits, but was not found to be essential for genome replication (Peh et al., 2004a). In addition the E1^E4 proteins from HPVs are also thought to be important for optimal genome amplification, but like CRPV E1^E4, the E1^E4 protein from HPVs are not thought to be essential for viral genome amplification (Nakahara et al., 2004; Wilson et al., 2004). Several HPV E1^E4 proteins, including 16E1^E4, have been shown to cause G2/M arrest when they are expressed at high levels in epithelial cells (Davy et al., 2002; Nakahara et al., 2002; Knight et al., 2004). This function of HPV E1^E4 has been proposed to create an environment that favours viral genome replication, but prevents cellular replication.

Two threonine residues located in the N-terminal domain of 16E1^E4 have been shown to be essential for causing E1^E4-mediated cell cycle arrest (Davy et al., 2002). These residues were also found to be required for the retention of active cyclin B1 and cdk 1 (i.e. hypophosphorylated) complexes in the cytoplasm (Davy et al., 2005). This suggests that these two events may be linked. Similarly a threonine residue in the N-terminal domain of HPV1 E1^E4 has been shown to be essential for causing E1^E4-induced cell cycle arrest by modulating the cyclin B1/cdk 1 complex (Knight et al., 2004). Although the mechanism of E1^E4-mediated G2 arrest is thought to be slightly different for 1E1^E4 than 16 E1^E4, in that 1E1^E4 was found to prevent the accumulation of cyclin B1 expression in cells.

Virion release

The differentiated cells in the superficial layer of the epithelium form a durable barrier that potentially can inhibit virion release. The E1^E4 protein is abundantly expressed in these layers, where it is thought it may be able to facilitate virion release from beneath the epithelial surface. This effect of E1^E4 is thought in part to be related to the common ability of all E1^E4 proteins to associate with and in the case of some E1^E4 proteins including 16E1^E4 also to collapse the keratin filament network (Doorbar et al., 1991; Roberts et al., 1993; Pray and Laimins, 1995; Wang et al., 2004).

The cytokeratins networks found in epithelial cells are assembled from pairs of monomeric acidic (type I) and basic (type II) keratin molecules and provide structural integrity to cells (reviewed in (Coulombe and Omary, 2002)). HPV16 E1^E4 has been shown to interact strongly with keratin 18, which is a type I keratin but only bound weakly to keratin 8 a type II keratin (Wang et al., 2004). The N-terminal 16 amino acids of 16E1^E4, which contains the (LLXLL) motif which is conserved amongst E1^E4 proteins from mucosal PV was found to be sufficient for keratin colocalisation in cell culture (Roberts et al., 1994; Roberts et al., 1997; Wang et al., 2004). However, both the N- and C-terminal domains of 16E1^E4 are required to disrupt keratin dynamics (Roberts et al., 1997; Wang et al., 2004). It is thought that the E1^E4 protein associates with keratins via its N-terminus and associates with itself via its C-terminus, in this way E1^E4 can cross-link keratins and sometimes cause their collapse. Keratin collapse has been shown to occur both *in vitro* and *in vivo* in 16E1^E4 expressing cells resulting in the formation of perinuclear bundles (Doorbar et al., 1991; Roberts et al., 1997; Wang et al., 2004). The disruption of keratin dynamics is thought to increase cell fragility and perhaps facilitate virion egress.

Differences in the expression of several cellular proteins in the cornified cell envelope (CCE) have been reported in 11E1^E4 and 59E1^E4 positive sections compared to E1^E4 negative sections (Brown and Bryan, 2000; Bryan and Brown, 2000; Brown et al., 2004). CCEs are formed by the cross-linking of proteins such as loricrin, involucrin, keratin and small proline rich proteins to form an insoluble durable barrier just inside the keratinocyte cell membrane. It has been suggested by

immunofluorescence studies that cells expressing 11E1^E4, do not contain loricrin or keratin 10, unlike normal uninfected epithelium. The altered expression patterns of the CCE proteins are thought to give rise to a thinner wall and to account for the morphological aberrations that are seen in HPV11-infected CCEs (Brown and Bryan, 2000). Similarly it has been shown in HPV16 and HPV1 lesions that differentiation-specific keratins are expressed at lower levels in E1^E4 positive cells and that in 1E1^E4 positive cells a reduction in loricrin and filaggrin expression was also observed, compared with neighbouring E1^E4 negative cells (Doorbar et al., 1997). It is tempting to speculate that the changes in cellular protein expression levels observed in E1^E4 positive cells are a direct result of E1^E4 association, but as only circumstantial evidence links reduction in cellular integrity with E1^E4 expression it still remains to be determined.

The ability of 16E1^E4 to induce apoptosis when it is expressed at high levels is also thought to facilitate virion release (Raj et al., 2004). HPV16 E1^E4 has been shown to colocalise with mitochondria following the collapse of the keratin network. This association between E1^E4 and mitochondria, severely reduces the membrane potential of the outer mitochondrial membrane. This it is thought may lead to the slow release of cytochrome c, and subsequent apoptotic cell death in the upper layers of the epithelium, which may in turn facilitate virion release.

Putative role of E1^E4 in the regulation of gene expression

A yeast 2-hybrid screen of 16E1^E4 with an epithelial cell cDNA library lead to the discovery of the ability of 16E1^E4 to interact with a novel DEAD box protein, named E4-DBP (E1^E4-DEAD box binding protein) and another cellular protein later identified as toperisomerase II β-BP1 (TOPBP1) (Doorbar, ; Doorbar et al., 2000). DEAD box proteins play a role in RNA processing, stability and export as well as biogenesis of ribosomes (reviewed in (Tanner and Linder, 2001)). The cellular function of E4-DBP is unknown, although an E4-DBP homologue which has been identified in *E.coli* is involved in the regulation of RNA stability (Iost and Dreyfus, 1994). E4-DBP is predominantly nucleolar, but shuttles in and out of the nucleus (Doorbar et al., 2000). E4-DBP was found to colocalise with E1^E4 in the cytoplasm of some cells in the upper stratified layer but colocalisation was not obvious in all

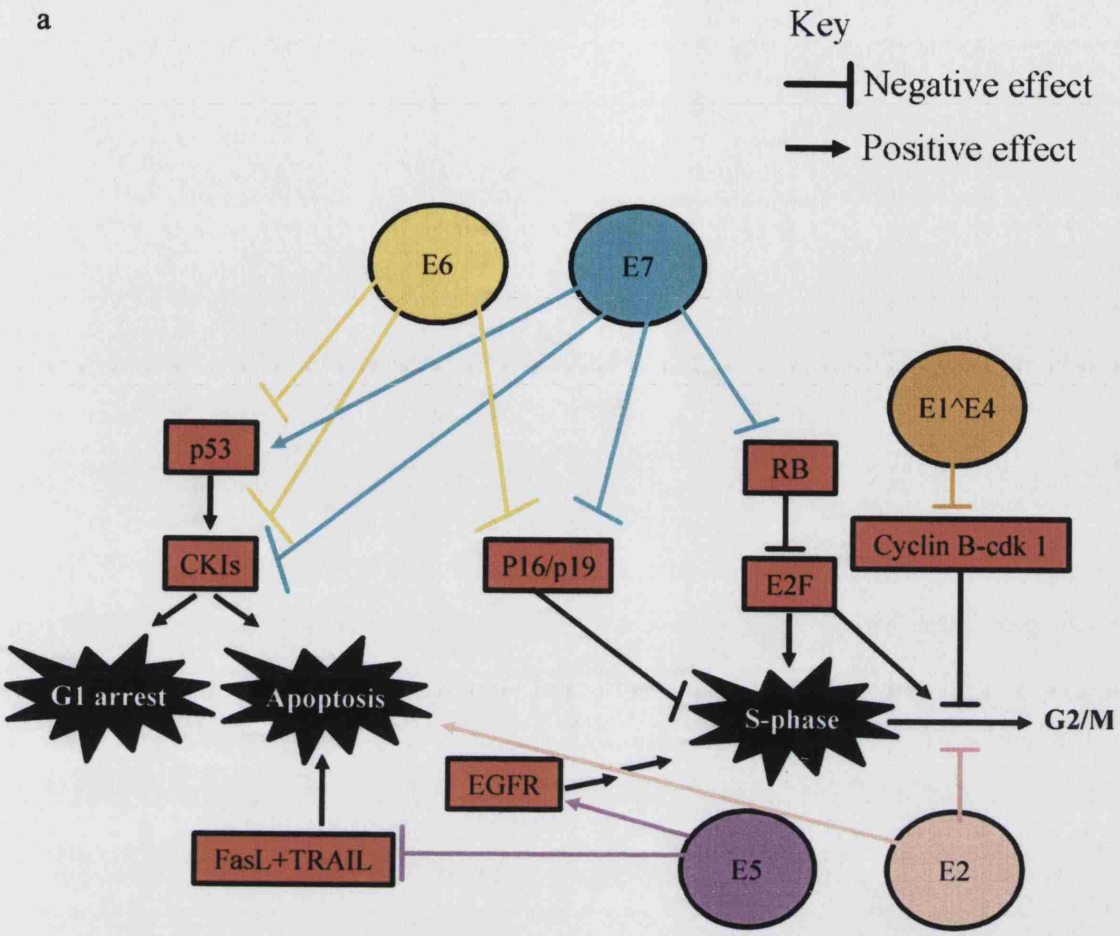
cells (Doorbar et al., 2000). The significance of 16E1^E4 binding to E4-DBP is not clear and does not seem to be a feature of all E1^E4 proteins, since HPV1 and HPV6 E1^E4 were unable to associate with E4-DBP in yeast 2-hybrid assays. Although it has been speculated that the E1^E4/E4-DBP interaction could be of importance for the post-transcriptional regulation of late HPV transcripts (Doorbar et al., 2000).

1.14 Cooperation between PV proteins

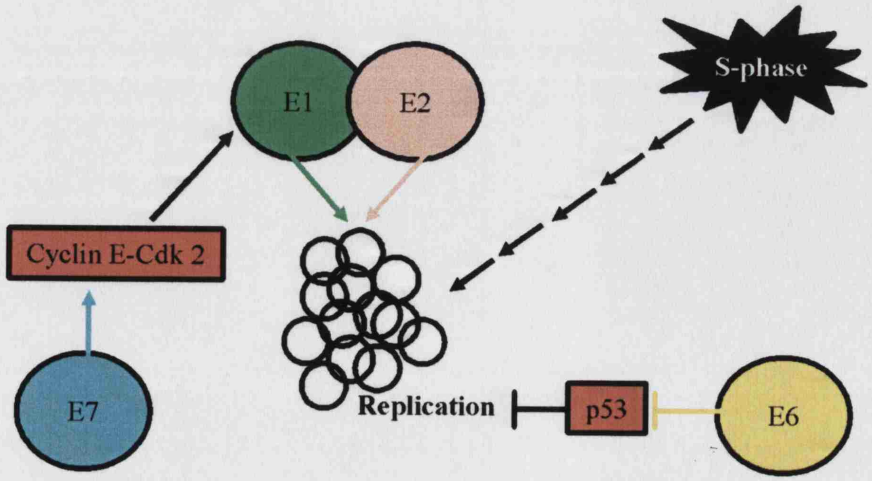
Several PV proteins have complementary roles in the life cycle. For example the functional cooperation between E6 and E7 proteins is essential, to create a balance between apoptosis and mitosis, ultimately stimulating cellular proliferation. The co-expression of other viral proteins, i.e. E1^E4 and E5, with E6 and E7, may help fine-tune the functions of E6 and E7, so as to create a cellular environment that is optimal for genome amplification (Figure 1.6a). Other HPV proteins have been shown to physically cooperate to mediate different stages of the life cycle, for instance E1 and E2, act together in viral genome amplification, and L1 and L2 interact with each other to package the PV genome (Figure 1.6b and c).

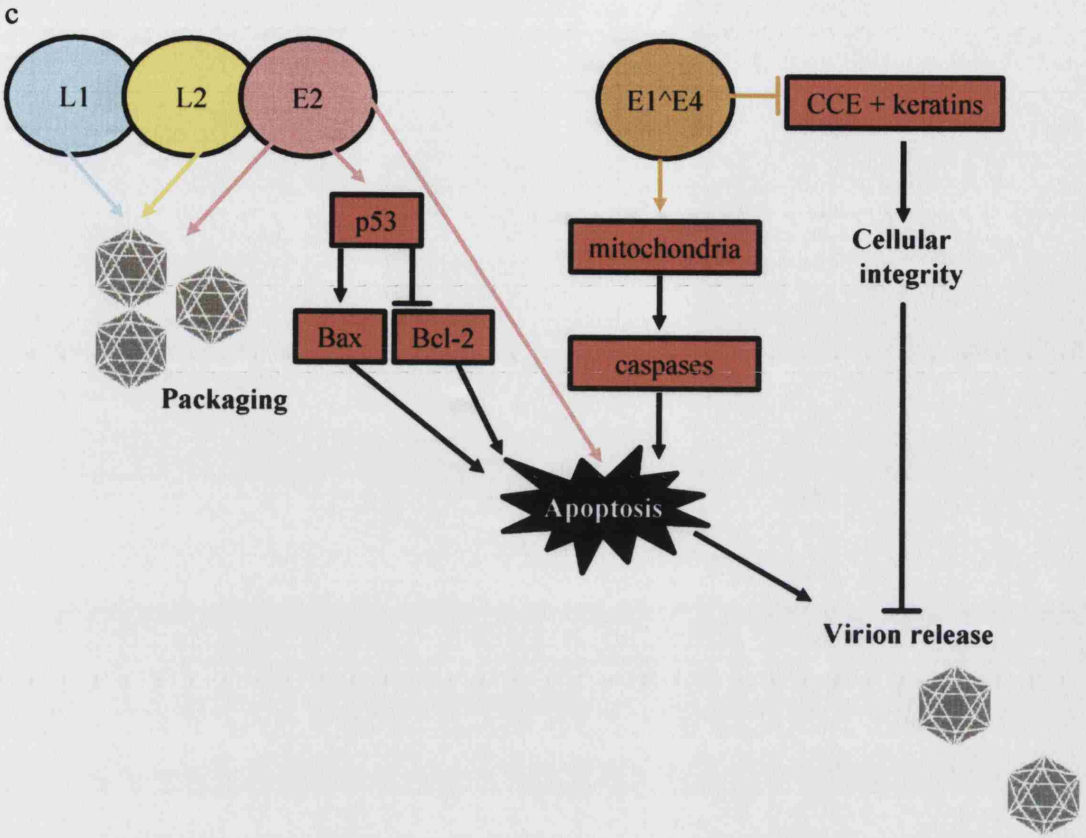
Figure 1.6 Schematic illustrations of cooperation between HPV proteins
The global "positive" and "negative" influences of different HPV proteins on cellular and viral processes are indicated.(a) The contribution of HPV E6, E7, E5, E2 and E1^E4 proteins, to apoptosis, G1 arrest and S-phase maintenance. (b) The contribution of E1, E2, E6 and E7 proteins, to viral replication. (c) The contribution of L1, L2, E2 and E1^E4 in infectious virion production and release.

a


Key
Negative effect
Positive effect
E6
E7
E1^E4
p53
RB
CKIs
P16/p19
Cyclin B-cdk 1
E2F
G1 arrest
Apoptosis
S-phase
G2/M
EGFR
FasL+TRAIL
E5
E2

b


S-phase
E1
E2
Cyclin E-Cdk 2
E7
Replication
p53
E6


c
L1
L2
E2
p53
Bax
Bcl-2
Packaging
E1^E4
CCE + keratins
mitochondria
caspases
Cellular integrity
Apoptosis
Virion release

1.15 Aims of the project

(A) Identify novel HPV16 E1^E4 binding partners

The primary objective of this thesis was to identify proteins that were found to associate with 16E1^E4 either directly or indirectly *in vitro*. The impetus for this project arose from several well-documented interactions of other HPV proteins with characterised cellular and viral proteins. The knowledge of these interactions has brought forward the understanding of the roles of other HPV proteins in the virus life cycle, and so it was hoped that this project would add to the understanding of the role of E1^E4 in the HPV life cycle.

(B) Identify the intracellular localisation of E1^E4 protein complexes

HPV16 E1^E4 has been shown to interact with four cellular proteins i.e. keratins, a DEAD box RNA helicase (E4-DBP), topoisomerase II β-BP1 (TOPBP1) and cyclin B1. 16E1^E4 has been shown to change the intracellular distribution of three of these proteins i.e. 16E1^E4 causes the collapse of the cytoplasmic keratin filament network both *in vitro* and *in vivo* resulting in the formation of perinuclear keratin bundles, and also causes the cytoplasmic retention of the RNA helicase protein (E4-DBP) *in vitro* and cyclin B1 *in vitro* and *in vivo*. Thus, it was envisaged E1^E4 could effect the intracellular distribution of possible novel interacting proteins, and so ideally the cellular localisation of all novel E1^E4 binding proteins were to be investigated.

(C) Investigate the functional significance of HPV protein interactions with E1^E4

Several HPV proteins have been found to cooperate during HPV infection to bring about the completion of the infectious virus life cycle, and so from the outset there was an intention to investigate the functional significance of any novel E1^E4-HPV protein interactions that were identified.

(D) To separate any novel effects of E1^E4 on the HPV life cycle from its G2-arrest function

It was important to be able to attribute any effects seen on any of the HPV life cycle functions that were to be investigated from those caused by E1^E4-mediated G2-arrest. As it was possible that several novel interactions may be identified in this study it seemed that the use of a characterised G2 arrest defective mutant would be useful in allowing the influence of the G2 arrest function of E1^E4 to be separated from the possible function(s) of any novel E1^E4 protein interactions that were being investigated.

2 Materials and Methods

2.1 Suppliers of reagents

Except where specified, or in the exceptions given below, reagents were obtained from Sigma-Aldrich Company Ltd., (Poole, UK), or BDH Laboratory Supplies, (Poole, UK).

Agarose, ammonium persulfate (APS), ethidium bromide (EtBr), sodium dodecyl sulfate (SDS) and N,N,N,N'-Tetra-methyl-ethylenediamine (TEMED) were obtained from Bio-Rad (Hemel-Hempstead, UK). FCS was obtained from Life Technologies (Consett, UK). ECL reagents, rainbow molecular weight markers, glutathione sepharose 4B and protein G Sepharose were obtained from Amersham Pharmacia Biotech, (Little Chalfont, UK). Ultrapure Protogel Acrylamide was obtained from National Diagnostics (Hull, UK). Long-Ranger gel solution was obtained from FMC Bioproducts (Rockland, USA). Complete protease inhibitor cocktail tablets (Cat no. 1697498) were obtained from Roche, (Lewes, UK).

2.2 Media and buffers

The following sterile media, solutions and buffers were obtained from NIMR media services and were prepared as follows:

NAME	COMPONENTS
Luria-Bertani Medium (LB)	1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % NaCl
LB agar	LB medium plus 2 % (w/v) Bacto agar
Salt-optimised broth + Carbon Medium (SOC)	2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.01 M NaCl, 0.025 M KCl, 0.01 M $MgCl_2$, 0.01 M $MgSO_4$, 0.02 M glucose
2 % (v/v) Trypsin versene, pH 7.8	0.8 % (w/v) NaCl, 0.02 % (w/v) KCl, 0.12 % (w/v) Na_2HPO_4, 0.02 % (w/v) KH_2PO_4, 0.01 % (w/v) EDTA, 0.13 % (w/v) trypsin, 0.001% phenol red.
Antibiotics [penicillin and streptomycin (Pen/Strep)]	0.6 % (v/v) penicillin, 1 % (v/v) streptomycin
1 x Phosphate Buffered Saline (PBS)	1% (w/v) NaCl, 0.025 % (w/v) KCl, 0.14 % (w/v) Na_2HPO_4, 0.025 % (w/v) KH_2PO_4
10 x Tris borate / EDTA (TBE)	12.1 % (w/v) Trizma base, 6.2 % (w/v) boric acid, 1.9 % (w/v) EDTA
20 x Standard Saline-Citrate (SSC)	3 M NaCl, 0.3 M Na-citrate, (pH 7)

2.3 Molecular biology techniques

2.3.1 Bacterial cell culture

2.3.1.1 *E.coli* strains

E.coli strains used are shown. Except where specified the strain used was DH5α.

STRAIN	GENOTYPE	SUPPLIER
DH5α	*deoR endA1, gyrA96, hsdR17(rk- mk+) recA1, relA1, supE44, thi-1, Δ(lacZYA-argFV169) φ80δlacZΔM15, F-, λ-*	Clontech
XL-1 blue	*recA1 endA1 gyra96 thi-1 hsdR17 supE44 relA1 lac [F' proAB laclqZΔM15 Tn10* (Tetr)]	Stratagene
BL21 (DE3) star	*F- ompT, hsdSB, (rB-mB-), gal dcm rne131* (DE3)	Stratagene
BL21 (DE3) pLysS star	*F- ompT, hsdSB, (rB-mB-), gal dcm rne131* (DE3), pLysS (CamR)	Stratagene

2.3.1.2 *E.coli* culture

Cells were grown in LB medium containing appropriate antibiotics to select for plasmid maintenance. The antibiotics used were ampicillin (100 μgml^{-1}), kanamycin (50 μgml^{-1}) and chloramphenicol (30 μgml^{-1}). Cultures were incubated at 37 °C, on agar, or in liquid medium at 37 °C, with shaking (220 rpm) unless otherwise specified.

2.3.1.3 *E.coli* glycerol stocks

E.coli was stored long term as glycerol stocks. Equal volumes of mid-log cultures and 50 % (v/v) glycerol solution were thoroughly mixed snap frozen on a dry-ice/methanol bath and then stored at –80 °C.

2.3.1.4 Preparation of electrocompetent *E.coli* (DH5α)

E.coli cells from 200 ml culture with an optical density at a wavelength of 600 nm (OD_{600}), ~0.5 were pelleted (6000 G, 15 mins, at 4 °C) and resuspended in 200 ml ddH_2O, 4 °C. The suspension was re-spun three times resuspending sequentially in 100 ml ddH_2O and then 4 ml of 10 % (v/v) glycerol and then in 200 µl 10 % (v/v) glycerol, all at 4 °C. Cells were used immediately or aliquoted and stored at –80 °C.

2.3.1.5 Transformation of plasmid DNA into *E.coli* by electroporation

Except where specified *E.coli*, was transformed with plasmid DNA according to the following protocol. Plasmid DNA, approximately 100 ng, was mixed with electrocompetent *E.coli* cells (50 µl) and electroporated (1.5 kV, 25 µF, 200 Ω) in 0.2

cm electrocuvettes using a Gene Pulser (Bio-Rad). Cells were allowed to recover by shaking at 37 °C in 1 ml of SOC for 45 mins. The cells were subsequently pelleted (3500 G, 20 secs) then resuspended in 200 µl of SOC, spread on LB agar plates containing selective antibiotics using asceptic technique and incubated overnight (O/N) at 37 °C.

2.3.1.6 Transformation of plasmid DNA into *E.coli* by heat shock

BL21 (DE3) star and where specified BL21 (DE3) pLysS star cells, were used for the preparation of glutathione S-transferase (GST) and Histidine-tagged proteins, because this *E.coli* strain carries a chromosomal copy of the bacteriophage T7 RNA polymerase gene which is under the control of the IPTG-inducible lacUV5 promoter. This enables the inducible expression of genes encoding GST and Histidine-tagged proteins cloned downstream of a T7 promoter in bacteria following transformation with T7 expression plasmids. Chemically competent bacteria were allowed to thaw on ice for 5 mins before being transferred to 15 ml polypropylene round bottom tubes (BD Biosciences). Purified plasmid DNA (1 µl) was added directly to the cells, which were then incubated on ice for a further 30 mins.

The tubes were then held for 30 secs in a 42 °C water bath before being returned to the ice for a further 2 mins, then SOC media (300 µl) was promptly added. Cells were allowed to recover by shaking at 37 °C in 1 ml of SOC for 45 mins. The cells were subsequently pelleted (3500 G, 20 secs) then resuspended in 200 µl of SOC, spread on LB agar plates containing selective antibiotics using asceptic technique and incubated O/N at 37 °C.

2.3.2 Molecular cloning

2.3.2.1 Purification of plasmid DNA from *E.coli*

Plasmid DNA was isolated from *E.coli* using Quantum Prep® Plasmid miniprep kit (Bio-Rad), and on a larger scale using the EndoFree Plasmid Maxi Kit (QIAgen), according to the manufacturer's instructions.

2.3.2.2 Quantitation of DNA

DNA concentrations were assayed using a 1201 UV-visible spectrophotometer (Shimadzu, Milton Keynes, UK). DNA solutions were diluted 1:100-1:500 and their absorbance at 260 nm measured in quartz cuvettes. DNA concentrations were calculated assuming an A_{260} of 1 is equivalent to 0.05 $mgml^{-1}$.

2.3.2.3 TBE agarose gel electrophoresis

PCR products and low MW DNA such as plasmid DNA were separated on 1.5, 1 and 0.8 % gels as specified, buffered with 1 x TBE, containing 1 μgml^{-1} EtBr. Samples were mixed with 10 x loading buffer (50 % (v/v) glycerol, 0.5 % (w/v) bromophenol blue, 0.4 % (w/v) xylene cyanol) and electrophoresis was carried out, at a constant voltage of 70 V, limiting to 200 mA. DNA bands were visualized by trans-UV illumination and compared to DNA markers (Invitrogen) of known molecular mass. Digital images were captured with a Kodak Image Station 440 and Digital Science 1D software (Perkin Elmer Life Sciences, Ltd., Cambridge, UK).

2.3.2.4 DNA digestion with restriction endonucleases

DNA was digested with restriction endonucleases at 37 °C, in 1 x optimal buffer, as detailed below and 1 % (v/v) bovine serum albumin (BSA). When testing for the presence of inserts, 0.5-5 µg of DNA was reacted with 5 U of enzyme in a total volume of 20 µl for 3 h. Preparative restriction digests for cloning utilized approximately 10 µg of plasmid DNA or the entire PCR product. These were reacted with 20 U enzyme in a total volume of 50 µl, O/N.

Restriction endonucleases (REs) and buffers

RE	BUFFER	SUPPLIER
BamHI	B	Roche
EcoRI	H	Roche
NcoI	H	Roche
XhoI	H	Roche
NheI	M	Roche
BglII	M	Roche

2.3.2.5 DNA purification from enzyme reactions

Enzymes and buffers used in restriction digests and phosphatase reactions were removed from the DNA sample, using the QIAquick PCR purification kit or the QIAquick Gel Extraction Kit (QIAgen), according to the manufacturer's instructions.

2.3.2.6 Polymerase chain reactions

PCR was used to amplify sequences for molecular cloning and to confirm the presence of insert. Primers were designed to be complementary to the target sequence but incorporate restriction sites and start codons where appropriate. PrimerSelect software (DNA Star, Madison, USA) was used to anticipate and minimize the formation of primer dimers, hairpin loops and sites of mis-priming. All primers were obtained from Oswel (Southampton, UK).

PCR reactions for molecular cloning and screening were assembled using the following reagents:

Template	100 ng of DNA
deoxy-nucleotides (dNTPs)	10 mM each
10 x PCR buffer (Invitrogen)	5 µl
$MgS0_4$, (Invitrogen)	1 mM
Forward primer	50 pmol
Reverse primer	50 pmol
Platinum® Pfx polymerase (Invitrogen)	2.5 U
Total volume (made up with ddH_20)	50 µl

PCR reactions were cycled on a Thermal Cycler 200 (GRI, Braintree, UK), except where specified, and according to the following parameters:

Step 1 94 °C, 5 mins

Step 2 94 °C, 1 min

Step 3 50 °C, 1 min

Step 4 68 °C, 1 min

Step 5 Go to step 1 x 29 cycles

Step 6 70 °C, 10 mins

Step 7 4 °C hold

2.3.2.7 Purification of PCR products

PCR products were purified from agarose gels using the QIAquick Gel Extraction kit (QIAgen) according to the manufacturers' instructions.

2.3.2.8 DNA sequencing

DNA sequencing reactions were carried out using the DNA Sequencing Kit (Applied Biosystems) and plasmid DNA as templates in the following reaction:

Big Dye™ Terminator Cycle Sequencing Reaction	4 µl
Half Big Dye™ buffer	4 µl
Template DNA	200 ng
Primer	5 pmol
Total volume (made up with ddH_20)	20 µl

Reactions were cycled according to the following parameters:

Step 1	96 °C, 10 secs
Step 2	50 °C, 5 secs
Step 3	60 °C, 4 mins
Step 4	Go to step 1 x 25 cycles
Step 5	4 °C hold

2.3.2.9 DNA sequencing sample precipitation

To remove unincorporated bases, the reactions were mixed with 50 µl 100 % EtOH and 2 µl of 5 M NaAc, pH 5.2 and incubated on ice for 10 min. The amplified products were pelleted (10,000 G, 15 mins). The pellets were then washed with 70 % EtOH and briefly re-spun, before the EtOH was carefully removed. The DNA pellets were dried and resuspended in 6 µl DNA sequencing sample loading buffer (100 µl formamide, 20 µl 25 mM EDTA, pH 8.0, 50 $mgml^{-1}$ dextran blue).

2.3.2.10 DNA sequencing gels

Polyacrylamide sequencing gels (5 ml 10 x TBE, 5 ml Long-Ranger gel solution, 18 g urea, 250 µl 10 % (w/v) APS, 25 µl TEMED and ddH_20 up to a total volume of 50 ml), were cast and assembled onto the ABI PRISM 377 DNA Sequencer (Applied Biosystems). The sequencing samples were denatured by incubation at 95 °C for 5 mins, loaded onto the gel and sequenced according to the manufacturer's instructions.

2.3.2.11 DNA sequence analysis

DNA sequence data was acquired using ABI Sequencing Analysis 3.3 software (Applied Biosystems) and sequences were analysed using Lasergene software (DNA Star, Madison, USA).

2.3.2.12 Vector preparation

Vector DNA, pGL3.Basic (Promega, Appendix II) or pET-28a(+) (Novagen, Appendix II) was digested with restriction enzymes as outlined. Restriction sites for directional cloning were chosen to be suitably far apart to ensure efficient double digestion. Adequate digestion was confirmed by separation on 1 % TBE agarose gels. Linearised vector was gel purified and quantitated.

2.3.2.13 Insert preparation

Inserts were obtained by PCR amplification using specific primer pairs. Restriction sites within the primers allowed generation of PCR products with cohesive ends to match those of the target vector. Digested PCR products were gel purified.

2.3.2.14 Ligation of insert into vector

Double digested, vector (0.5 μg) was ligated to insert in equimolar quantities, with 40 U T4 DNA ligase (NEB) and 2 μl 10 x ligase buffer (NEB). Reactions were incubated in a total volume of 20 μl at 15 °C for 16 h.

2.3.2.14 Detection of positive clones

E.coli, were transformed with 2 μl ligation mix and the resulting colonies were cultured in liquid medium prior to plasmid DNA isolation by miniprep. Plasmid DNA was digested with the suitable restriction enzymes to indicate the presence of insert in the correct orientation. The PCR products were separated on TBE agarose gels and the presence of correctly sized bands used to indicate positive clones.

2.3.2.14 Cloned constructs

The cloning primers for this study are shown in Appendix III. Note the primers and vectors used to clone pET.16E1^E4M2 through to pET.16E1^E4M12 were the same as those used for pET.16E1^E4. The yeast two-hybrid vector, pGBT9 (Clontech) containing wild-type (WT) and the appropriate deleted E1^E4 sequences (Doorbar et al., 2000) were used as template for the cloning of E1^E4 and E1^E4 mutants into pET-28a(+). The 18E1^E4 sequence was amplified from pGEX.18E1^E4 (A gift from Dr, Heather Griffin, MRC Centre for Protein Engineering, Cambridge). The cloning of pET.16E1^E4 and pET.18E1^E4 was done by Dr. Pauline McIntosh (NIMR) and the cloning of pET.E1^E4M1-M14 was done with the assistance of Dr. Deb Jackson (NIMR).

The vector pET-23b(+) (Novagen) containing the E1, E2 and E4 ORFs nt 865-3852 (all nt numbers correspond to the HPV16, W12E genome (Accession no. AF125673),

unless specified). The W12E genome was isolated from the W12E cell line which was derived from a HPV-infected patient, and appears to be identical to the sequenced HPV16 clone (Stanley et al., 1989; Flores et al., 1999). The W12E genome was used as the template for the PCR of WT cassette and the vector pET-23b(+) containing the E1, E2 and E4 ORFs nt 865-3852, with two stop codons introduced into the E4 ORF was used as template for amplification of the mutant cassette. Stop codons were introduced into the E4 ORF of the HPV16 genome, using the Quick-change site-directed mutagenesis kit (Stratagene) with the primers specified in Appendix III. The mutagenesis and sub-cloning of the WT and mutant cassettes into pET-23b(+) was performed by Dr. Woei Ling Peh (NIMR) (Peh, 2002). WT and mutant cassettes were subsequently transferred from this vector to pIRESeGFP2 (pIRES, Clontech, Appendix II) by Dr. Heather Griffin (MRC Centre for Protein Engineering, Cambridge), using the specified primers.

The template that was used for the amplification of the LCR was the W12E genome, which was cloned into the vector pSp64 via the *BamHI* site at nt 6150, the construct was called pSpW12 and was a kind gift from Dr. Margaret Stanley, The Stanley Laboratory, Cambridge University, Cambridge.

2.4 Cell culture techniques

2.4.1 Cell lines

2.4.1.1 SiHa cells

SiHa cells are a transformed cervical epithelial cell line derived from a tumour containing integrated HPV16 genome (Friedl et al., 1970). SiHa cells constitutively express E6/E7 but lack E1^E4 expression due to loss of the E4 ORF. This cell type gives high efficiency infection with recombinant adenoviruses (rAds).

2.4.1.2 HEK293 cells

HEK293 is a human embryonic kidney cell line transformed with a fragmented adenovirus type 5 (Ad5) genome that constitutively expresses the adenovirus, E1 protein. For this reason, HEK293 cells can be used to complement Ad5ΔE1 recombinant vectors to facilitate the production of infectious adenovirus particles. Optimum virus production occurs in early passage cells, and the cells used in the experiments described here were cultured for no more than 20 passages (post arrival from the European Cell Culture Collection).

2.4.1.3 Cos-7 cells

COS-7 cells are a simian virus 40 (SV40) transformed, African green monkey kidney cell line (Gluzman, 1981). This cell line gives a high efficiency of transfection, especially with the Effectene transfection reagent (QIAgen).

2.4.1.4 MCF-7 cells

MCF-7 cells are a multi drug resistant breast cancer cell line which has retained an active p53 gene (Devarajan et al., 2002). This cell line was used for high levels of expression of HPV16 E2 protein, with a recombinant adenovirus expressing E2 (rAd.E2) (Bechtold et al., 2003), as E2 does not negatively regulate the cell cycle in this cell line as it does in some others (Bellanger et al., 2001).

2.4.1.5 C33a cells

C33a cells are a cervical epithelial carcinoma cell line which is not HPV transformed. C33a cells lack functional p53 gene due to an inactivating mutation at codon 273 (Scheffner et al., 1991). This cell line was used in transcription assays, as it is a cervical epithelial cell line so it is thought to contain a similar range of transcription factors and regulatory machinery as normal cervical epithelium. In addition as C33a cells do not contain HPV DNA, no endogenous E2 binding sites are present in the cellular genome, which could sequester both cellular and artificially expressed viral transcription factors.

2.4.2 Routine cell passage

Cell cultures were maintained in complete medium consisting of Dulbecco's modified Eagles medium (DMEM) supplemented with 10 % (v/v) fetal calf serum (FCS) and 1 % (v/v) Pen/Strep in a 5 % CO_2 environment at 37 °C. On reaching confluency cells were removed from their flasks by first washing in PBS and then incubation in 1 ml of 2 % (v/v) trypsin-versene at 37 °C for ~5 mins. The flask was then lightly knocked to detach the cells from the base of the flask. The trypsin was neutralized with 4 volumes of complete medium and cells were plated into fresh culture containers.

2.4.3 Seeding cells

Media was removed from cells cultured in T-75 flasks, and the cells were rinsed with PBS twice. The remaining cells adhered to the interior of the tissue culture flask were incubated with 2 ml of 2 % (v/v) trypsin-versene solution for ~5 mins at 37 °C. The flask was then lightly knocked to detach the cells from the base of the flask. The flask was then flooded with 10 ml of complete media. The cells in solution were counted on a haemocytometer, using a phase contrast light microscope (Nikon Eclipse TS100, Surrey, UK) to estimate the number of cells in 1 ml of media, enabling cells to be plated at an appropriate density as specified in the following table.

CULTURE FORMAT	NO. OF CELLS SEEDED THE DAY PRIOR TO TRANSFECTION
96-well plate	2×10^4
24-well plate	5×10^4
6-well plate	2.5×10^5
90 mm plate	5×10^5

2.4.4 Long term storage of cells

Cells were cultured until confluent and then ~$1x10^6$ cells were pelleted (2,500 G, 5 mins) and then resuspended in 1 ml of freezing medium (90 % (v/v) FCS, 10 % (v/v) DMSO) and transferred to a cryotube. This was stored at -80 °C, O/N wrapped in 12-15 layers of tissue paper to slow down the freezing of the cells, before being transferring to liquid nitrogen. When required the cells were rapidly defrosted at 37 °C and added to 10 ml complete medium. The cells were pelleted as above, resuspended in 15 ml of complete medium and transferred to a T75 tissue culture flask.

2.4.5 Serum starvation of cells

C33a cells were starved of serum to try and cause G1 arrest of the cell cycle. This was done by simply removing complete media 36 h prior to harvest and replacing it with just DMEM for the remainder of the incubation.

2.4.6 Monoclonal antibody production

TVG402 was produced from a hybridoma cell line (Doorbar et al., 1992), which was maintained in complete media. Following the recovery of the TVG402 hybridoma cell line from long term storage, the cells were passaged at least 6 times before being used for antibody production. The cells were then grown until they were over-confluent (~ 4-7 days after confluence was reached). Subsequently, the supernatant containing ~20-50 μg/ml of antibody was collected and then briefly centrifuged to remove cell debris (1000G, 10 mins). The cell culture supernatant was then filtered

through a 0.2 μm filter, and 0.02 % (v/v) sodium azide (NaAz) was added to the solution, to prevent bacterial growth and was stored at 4 °C.

2.4.7 Mitochondria labelling

MitoTracker® Red CMXRos (Molecular Probes) was diluted in 1 ml of complete media to a final concentration of 250 nM. Living cells seeded on 13 mm coverslips were incubated in this staining solution for 30 mins at 37°C. The cells were then washed three times with PBS prior to formaldehyde fixation and double staining by immunofluorescence.

2.4.8 Transfection of cultured cells

Commercial kits were used to transfect cultured cells, 24 h after the cells were seeded. The Effectene transfection kit (Qiagen) was used according to manufacturer's instructions to transfect cells on 13 mm coverslips in 24-well dishes, for immunofluorescence and for adenovirus stock preparation. The Profection transfection kit (Promega) was used to transfect cells for functional assays and Western blotting in 6-well tissue culture dishes. Briefly 8-20 μg of DNA was diluted in 300 μl of ddH_2O to which 37 μl of 2 M calcium chloride ($CaCl_2$) was added and the mixture was vortexed for 5 secs. Next 300 μl of 2 x Hepes Buffered Saline (HBS) was added to the sample, which was vortexed for 5 secs, then incubated at R/T for 20 mins. The precipitating mixture was added to a single well of a 6-well dish in 4 ml of complete media. Approximately, 16 h later the media covering the cells was exchanged for fresh complete media. The source of all the vectors that were used in this study but are not described in Section 2.3.2 are given in Appendix IV.

2.4.9 Adenovirus culture

2.4.9.1 Preparation of high titre adenovirus stocks in HEK293 cells

Adenovirus stocks were prepared by initially transfecting HEK293 cells with recombinant adenovirus plasmids. Transfected cells were incubated for a further 7-10 days post transfection at 37 °C to allow virus particles to be formed. Cells were then

scraped from the flasks, pelleted (1250G, 5mins) and resuspended in 2 ml of 1 % (v/v) FCS/PBS. The cells were cracked open by freeze/thawing three times with dry ice/MeOH and a 37 °C water bath. The cells were vortexed vigorously between freezing steps and finally the debris was pelleted (10,000 G, 15 mins). The crude supernatant was used to infect approximately double the number of cells. To obtain high titres of recombinant adenoviruses multiple rounds of amplification were used.

For routine propagation of rAds, 16 x T75 tissue culture flasks of HEK293 cells were grown to ~70 % confluency and then infected with high titre recombinant adenovirus containing supernatant. The cells were then incubated for 2-3 days, when comet shaped clearings could be seen in the culture flasks. The cells were then harvested when at least 1/3-1/2 of the cells were detached, which was ~ 5 days post infection. The cells were then cracked open as already described. To increase infection rates cells were infected with viral supernatants diluted in DMEM containing antibiotics and supplemented with 5 % (v/v) FCS.

2.4.9.2 Purification of rAds

The adenovirus-containing supernatants were further purified on CsCl density gradients prior to use for recombinant protein expression in SiHa and MCF-7 cells. CsCl gradients were prepared in pollyallomer tubes (14 x 89 mm, Beckman, High Wycombe, UK), by first pipetting 4 ml of CsCl with a density of 1.45gml^{-1} and then slowly pipetting on top, 6ml of CsCl with a density of 1.2gml^{-}1. The supernatants were then loaded onto the gradient and then centrifuged at 32,000 rpm for 1 h at R/T.

An opaque band was produced by centrifugation in the middle of the gradient which contained purified rAd; this was carefully removed using a 23 gauge needle and a 1 ml syringe. The rAd solution was then aliquoted and stored at -70 °C before use the rAd was titrated using the cytopathic effect (CPE) method.

2.4.9.3 CPE titration

The purified adenoviruses were titrated using the CPE endpoint assay (Precious and Russell, 1985). Briefly, the adenovirus solution was serially diluted (6 x 10 fold

dilutions, followed by 16 x 2 fold dilutions). Each dilution was added in triplicate to a 96 well plate containing HEK293 cells seeded at 2 x 10^4 cells per well. This assay was repeated in triplicate and incubated for 6 days at 37 °C. The titre was estimated based on the minimum adenovirus dilution, at which 2/3 of the wells were infected.

2.4.9.4 Adenovirus infection of cells

Cells were infected with rAd at a multiplicity of infection (MOI) of 100. Recombinant adenoviruses diluted in DMEM containing antibiotics and supplemented with 5 % (v/v) FCS were incubated with cells for at least 16 h prior to harvesting the cells. The source of all the rAds that were used in this study but are not described in Section 2.3.2 are given in Appendix IV.

2.4.10 Radioactive-labelling of cells

SiHa cells were seeded at a density of 5x10^5 cells/90 mm dish a total of 6 dishes were seeded per an experiment. Three dishes were infected with a recombinant adenovirus expressing E1^E4 (rAd.E1^E4) (Davy et al., 2002) and the remaining three dishes with a recombinant adenovirus expressing β-galacosidase (rAd. β-gal) (Davy et al., 2002). At 19 h post infection media was removed from the cells, which were then rinsed twice with PBS. To each plate 3 ml of Methionine and Cysteine free DMEM media (ICN Biochemicals, High Wycombe, UK) supplemented with 10 % (v/v) dialysed FCS (Perbio Science) was added and the cells were returned to the incubator for 2 h. Subsequently, 0.5 ml of the incubation media was removed from each plate and used to dilute 44 µl (18.5 MBq) of Redivue Promix ^{35}S-Cysteine,-Methione (Amersham Pharmacia Biotech), and the radioactive media was returned to each plate drop-wise. The plates were briefly swirled and then returned to the incubator for a further 3 h. Cells were then harvested.

2.5 Cell staining and analysis

2.5.1 Immunofluorescence techniques

2.5.1.1 Fixation and permeablilization of cultured cells

Cells were grown on glass coverslips (13 mm diameter) in individual wells of a 24-well plate and where specified either transfected or infected with vectors expressing recombinant proteins. The cell culture media was removed from the wells and the coverslips were washed three times by swirling in PBS at 5 minute intervals. The cells were then fixed in 5 % (v/v) formaldehyde for 5 mins before being washed again. This step was repeated five times. Cells were then permeabilized by incubation of the coverslips in cold 0.5 % (v/v) NP40/10 % (w/v) sucrose/PBS for 10 mins. Finally, the cells were blocked in 2 % (w/v) BSA/0.01 % (v/v) NaAz/ PBS, for at least 30 mins, at R/T or at 4 °C, O/N.

2.5.1.2 Immunostaining of cultured cells

BSA blocked cells were incubated with primary unconjugated antibodies diluted in 0.1 % (w/v) FCS/PBS, at 37 °C for 1 h and then were washed five times in PBS at 5 minute intervals. Cells were then incubated with Alexa Fluor® 488 or Alexa Fluor® 594 (Molecular Probes) conjugated secondary antibodies and 1 μgml^{-1} of 4'-6' diamidino-2-phenylindol DAPI diluted in 0.1 % (v/v) FCS/PBS, at 37 °C for 1 h and then washed five times with PBS at 5 minute intervals. DAPI, forms a fluorescent complex with DNA, and is thus used as a nuclear stain in these immunofluorescent studies.

For double staining of E2 and E1^E4, E2 was stained for first, as above and then Alexa-488 conjugated TVG405 diluted in 0.1 % (v/v) FCS/PBS, was subsequently incubated with the cells for 1 h, and then washed at 5 minute intervals with PBS. In cases where E1^E4 was detected using an unconjugated antibody, the primary and secondary antibody incubation and washing steps were repeated but with different antibodies and in all cases DAPI was incubated with the cells on the final antibody incubation step. Finally, the coverslips were rinsed briefly with ddH_20 then inverted onto 4 µl of citifluor (Agar Scientific, Stanstead, UK) on a microscope slide.

Immunostaining of cultured cells was performed with the assistance of Dr. Deb Jackson.

2.5.1.3 Immuno-detection of formalin-fixed tissue and raft sections

Formalin-fixed HPV16 infected low grade lesions and HPV16 genome transfected, raft sections of the near-diploid human keratinocyte cell line (NIKS) (Allen-Hoffmann, 2000) mounted onto electrostatically charged microscope slides were obtained from Karl Sotlar (University of Turbingen, Turbingen, Germany) and Paul Lambert (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin) respectively, the latter was produced by a PhD student Tomomi Nakahara in the laboratory.

Sections were placed in a slide rack and dewaxed by incubation in xylene for 10 mins and then the sections were rehydrated by incubation in 100 %, 70 %, 50 % and 30 % ethanol solutions in series, each incubation was for 2 mins. The slide rack was then briefly submerged in water, before being transferred to a microwavable container containing freshly prepared citrate buffer (0.01 M citric acid, pH 6), the container was loosely covered and then microwaved at 800 Watts for 18 mins. The slides were then left to cool for 15 mins at R/T in the citrate buffer. The citrate buffer was slowly exchanged for ddH_20 by removing ~20 % of the incubating citrate buffer and replacing it with ddH_20, this step was repeated 5-6 times.

The slides were then incubated in PBS for 30 mins, while slide chambers were prepared by placing wet tissue paper inside them. The perimeter around the tissue section was circled with an ImmEdge wax pen (Vector Laboratories Incorporated, USA) to form an enclosure to minimise the amount of staining reagents needed to cover the section. The sections were blocked with 5 % (v/v) goat serum/ PBS, which was added drop wise onto the marked section and left to incubate for 20 mins at R/T. The slides were then gently placed on their sides and tapped onto a tissue to remove the goat serum.

The E2C antibody provided by Prof. Norman Maitland (University of York, UK) was first preabsorbed with acetone powder of human skin (kindly provided by Dr. Woei Ling Peh, NIMR) for 30 mins at 4 °C. The mixture was then centrifuged briefly for

30 secs at 2000G and the supernatant was removed. All the primary antibodies used were diluted as specified were then added onto the sections and incubated at 37 °C in the humidified slide chambers for 1 h. The antibodies were then removed and the sections washed thoroughly by pipetting PBS over the sections at least 6 times before incubating the sections at R/T in PBS for 10 mins followed by a further 2 washes. Secondary antibody diluted in PBS as specified was added to the sections and left to incubate at 37 °C for 30 mins in the slide chamber. The slides were washed as before and if double staining the antibody incubation steps were also repeated but with different antibodies, in cases where Alexa-488 conjugated TVG405 was used there was no need for secondary antibody incubations. DAPI, diluted to 1 μgml^{-1} DAPI in PBS was added to the sections and left to incubate for 10 mins at R/T. The slides were washed as before in PBS before being rinsed briefly with ddH_20, the cells were then mounted with rectangular coverslips and citifluor and viewed by fluorescent microscopy.

2.5.1.3 Antibodies used for immunostaining

The following antibodies were used at the specified dilutions for immunofluorescent staining. The fluorophore conjugated TVG405 antibody (Doorbar et al., 1992) is a synthetic Fab antibody which was used to detect the E1^E4 protein. TVG405 was purified by Ni-NTA affinity chromatography and directly labelled using either an Alexa Fluor® 647 or an Alexa Fluor® 488 labelling kit (Molecular Probes) by Dr. Deb Jackson (NIMR). The conjugated antibodies were used at a dilution of 1 in 1000 or 1 in 100, respectively. All secondary polyclonal antibodies used were affinity purified cross-adsorbed polyclonal species, produced by Molecular Probes.

Primary antibodies:

Target	Antibody	Dilution	Antibody type	Source
HPV16 E2	TVG261	1:2	Mouse monoclonal antibody, in tissue culture supernatant	(Hibma et al., 1995)
HPV16 E2	TVG271	1:2	Mouse monoclonal antibody, in tissue culture supernatant	(Hibma et al., 1995)
HPV16 E2	Anti-E2C terminus	1:100, 1:1000	Rabbit polyclonal antiserum	(Maitland et al., 1998)
HPV16 E1^E4	TVG402	1:5	Mouse monoclonal antibody, in tissue culture supernatant	(Doorbar et al., 1992)
HPV16 E1^E4	Anti-MBP16E1^E4 (Rabbit G)	1:250	Rabbit polyclonal antiserum	(Doorbar et al., 1997)
HPV16 E1	E1-N1	Neat, 1:100	Mouse monoclonal antibody, in tissue culture supernatant	(Masterson et al., 1998)
Pan-Cytokeratins	F 0397	1:100	Purified mouse monoclonal antibody	(Sigma)

Secondary Antibodies:

Target	Antibody	Dilution	Species	Conjugated fluorophore
Mouse Ig	A11029	1:100	Goat	Alexa Fluor® 488
Mouse Ig	A11032	1:200	Goat	Alexa Fluor® 594
Rabbit Ig	A11034	1:150	Goat	Alexa Fluor® 488
Rabbit Ig	A11037	1:200	Goat	Alexa Fluor® 594

2.5.1.4 Microscopy

Cells were observed on a fluorescent Labophot II microscope (Nikon, Kingston upon-Thames, UK). Digital images were captured with a SenSys monochrome camera and IP Lab imaging software (Roper Scientific, Marlow, UK). Fluorescent signals were overlaid by computer assistance.

2.5.2 Flow cytometric analysis (FACS)

2.5.2.1 Preparation of C33a cells for flow cytometric analysis

Adherent cells for flow cytometry were harvested with 1 mM EDTA/2 % (v/v) trypsin-versene/PBS, washed with PBS and then resuspended in 100 µl of cold 70 % EtOH. The cells were pelleted (1250 G, 5 mins) and 5 x 10^5 cells were resuspended in

100 µl nonidet P-40 flow cytometry buffer (0.1 % (v/v) Nonidet P-40/0.1 % (w/v) BSA/PBS). The cells were incubated for 30 mins at R/T in the dark then pelleted as above. The cell pellet was resuspended in 100 µl Nonidet P-40 flow cytometry buffer supplemented with 100 μgml^{-1} RNase A and incubated for 20 mins in the dark. The cells were pelleted as above then resuspended in 250 µl of propidium iodide in PBS (12.5 μgml^{-1}) and then stored at 4 °C.

2.5.2.2 Flow cytometric analysis of C33a cells

Propidium iodide labelled cells were analysed by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, USA). Propidium iodide fluorescence was detected by the FL-3 (red) absorbance. Data was acquired for 1 x 10^4 cells using CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, USA) and represented as a dot-plot. The data was acquired and analysed using WinMDI software. The FL-3 values of the total cell populations were displayed as histograms.

2.6 Biochemistry Techniques

2.6.1 Recombinant protein production in bacteria

2.6.1.1 Constructs used for fusion protein expression

The following plasmids were used in this work pGEX.16E1, pGEX.16E2, pGEX.16E5, pGEX16.E6, pGEX.16E7, pGEX.16L1, pGEX.16L2 pGEX.16E1^E4, pGEX.18E2. The source of all these vectors is given in Appendix IV. The pET.16E1^E4 and pET.18E1^E4 and pET.16E1^E4 mutants which were also used encode his-tagged proteins are described in section 2.3.2. In all cases except pGEX.16L2, BL21(DE3) star cells were transformed with plasmid DNA, in the case of pGEX.16L2 BL21(DE3) pLysS star *E.coli* were used.

2.6.1.2 Equilibration of Glutathione, BD Talon and Amylose beads

Glutathione sepharose 4B (Amersham Pharmacia Biotech), Amylose resin (NEB) and BD Talon sepharose beads (BD Biosciences), were similarly treated to remove 20%

ethanol storage buffer. Briefly 100-500 µl of the sepharose resins were centrifuged (1000 G, 15 secs) the supernatants removed and the pelleted beads resuspended in 1 ml Net-N buffer (20mM Tris HCl (pH8), 100mM NaCl, 1mM EDTA, 0.5% (v/v) NP40, 25 mM protease inhibitor cocktail) or Urea buffer (8M Urea, 0.1M Monobasic NaH_2PO_4, 0.01M Tris-HCl, pH 7) respectively and then centrifuged again as before and the supernatant disguarded. This washing process was repeated 3 x, before the beads were finally resuspended as 50 % (v/v) slurry in 100-500 µl Net-N or Urea buffer (pH 7), respectively.

2.6.1.3 Induction of Fusion Proteins in *E.coli*

Cultures for producing fusion proteins were started by freshly streaking glycerol stocks of transformed bacteria onto selective LB agar and incubating O/N at 37 °C. Single bacterial colonies were subsequently scraped from the plate and transferred to 5 ml of selective liquid LB media and grown O/N, as described in section 2.3.1.2.

The following day the cultures were diluted into 500 ml of selective liquid LB media and allowed to grow, where specified LB media was supplemented with 0.5 M sorbitol and 2.5 mM betaine to reduce precipitation of proteins into insoluble aggregates (Blackwell and Horgan, 1991). The OD_{600} of the cultures were monitored at regular intervals, when the ODs reached values specified, the cultures were induced with IPTG and then returned to the incubator, under conditions as specified (section 2.6.1.6). Post induction the cultures were centrifuged (10,000 G, 20 mins), the supernatants were discarded and the pellets processed immediately or stored at -80 °C, until further use.

2.6.1.4 Maltose binding protein (MBP) and glutathione S transferase (GST), fusion protein extraction from *E.coli*

Fusion proteins were extracted from bacterial cell pellets. Standard extraction conditions were as follows, bacterial pellets of 500 ml cultures were resuspended in 50 ml of Net-N buffer in a glass beaker packed with ice, the bacteria were sonicated using a probe sonicator (Branson Digital Sonifier®), set at 34 Watts output for 3 mins

with 15 sec pulses. For extraction of GSTL2 from bacterial lysates, sonication was performed for only 1 min.

The bacterial lysates were then centrifuged (10,000 G, 20 mins), the clarified supernatants produced were then transferred into 50 ml falcon tubes to which 100-500 µl of equilibrated glutathione or amylose sepharose beads were added and then the tubes were rotated at 4 °C for 2 h. Next, the beads were pelleted by centrifugation (2000 G, 5 mins) and then resuspended in Net-N buffer and then repelleted by centrifugation (2000 G, 5 mins), this wash step was repeated 5 times. The beads with bound GST fusion proteins were finally resuspended at 50 % (v/v) in PBS-A/0.02 % (v/v) NaAz and then stored at 4 °C, for 2-6 weeks, until further use.

This protocol was adapted for extracting insoluble GST fusion proteins, such as GSTE1 and GSTE5. Basically the bacterial pellets were resuspended in ionic lysis buffer (1.5 % (w/v) N-Lauroyl scarosine (sarkosyl), 5mM DTT, 20mM Tris HCl (pH8), 100mM NaCl, 1mM EDTA), prior to sonication (Frangioni and Neel, 1993) instead of Net-N buffer. Post centrifugation of the bacterial lysate, 2% (v/v) of a non-ionic detergent Trition-X100 was added to the supernatant prior to mixing with beads to sequester sarkysol away from the proteins, allowing recombinant proteins to bind to beads.

2.6.1.5 His-tagged protein extraction from *E.coli*

His-tagged E1^E4 proteins were extracted from *E.coli*, as follows. Bacterial pellets of 250 ml cultures were resuspended in 20 ml of urea buffer (pH 7), the bacteria were sonicated on ice using a probe sonicator (Branson Digital Sonifier®), set at 34 Watts output for 2 mins with 15 sec pulses. The bacterial lysates were then centrifuged (10,000 G, 20 mins), the clarified supernatants produced were then added to TALON 2 ml gravity flow columns containing 100 µl of washed BD Talon beads (BD Biosciences) and left to drain through the column at R/T. The column was washed 5 x with 10 ml of urea buffer (pH 7). The purified his-tagged proteins were eluted from the beads in the columns with 3 x 1 ml of urea buffer (pH 4) and collected in 1.5 ml microcentrifuge tubes.

2.6.1.6 Conditions used for growing fusion proteins

Fusion Protein	Media	OD_{600}	Temp of Induction (°C)	[IPTG] (mM)	Induction Time (h)
GST/MBP	LB AMP	0.4-0.6	37	0.3	2
GST16E1	LB AMP + 0.5M SORBITOL + 2.5mM BETAINE	0.5-0.6	27	0.3	6
GST16E2 GST18E2	LB AMP	0.6	30	0.5	3.5
GST16E1^E4 MBP16E1^E4	LB AMP	0.4-0.6	30	1 0.2	2
GST16E6	LB AMP + 0.5M SORBITOL + 2.5mM BETAINE	0.4	30	0.1	4
GST16E7	LB AMP	0.4-0.6	30	0.5	2
GST16L1	LB AMP	0.7	30	0.2	4
GST16L2	LB AMP	0.5-0.7	23	1	1
His-16E1^E4 His-18E1^E4	LB AMP	0.7	30	0.2	3

2.6.1.7 Quantitation of protein

Protein concentrations were assayed against a blank of urea buffer using a 1201 UV-visible spectrophotometer (Shimadzu, Milton Keynes, UK). Protein samples were diluted in urea buffer pH 7 to 1:50-1:100 in a quartz cuvette and their absorbance at a wavelength of 260 nm, 280 nm and 320nm measured. Protein concentrations were calculated using the following formula.

$$[\mu g/ml] = \left(\frac{OD280 - OD320}{\text{Extinction Co-efficient}} \right) \text{x dilution factor x } 10^{-6} \text{ x MW}$$

2.6.2 *In vitro* protein binding and analysis methods

2.6.2.1 Harvesting cells

Cells were harvested by first removing the media and then rinsing the adherent cells twice with PBS. Next, the cells were incubated with 1 volume of 1mM EDTA/2 % trypsin-versene/PBS at 37 °C for ~10 mins. Subsequently, the majority of cells

floated off the plates, remaining adhered cells were washed off the plates using forceful pipetting with the incubation solution.

The cell suspensions were then transferred to 15 ml falcon tubes and centrifuged (1250 G, 5 mins). The supernatants were discarded and the cell pellets resuspended in PBS prior to centrifugation as before. Finally, the cell pellet was processed immediately or was snap frozen on a dry-ice/ethanol bath and then stored at -70°C until further use.

2.6.2.2 Protein extraction

Proteins were sometimes extracted from cells using denaturing buffers. Simply cells were resuspended and incubated on ice for 20 mins in RIPA buffer (150 mM NaCl, 1 % (v/v) NP40, 0.5 % (w/v) deoxychloric acid, 0.1 % (w/v) SDS, 50 mM Tris, pH 7.5) or 2 % (w/v) SDS/PBS. The samples were then briefly centrifuged before further use.

2.6.2.3 Cell fractionation

SiHa cells were fractionated according to their solubility in different detergents. Briefly, 90 mm dishes were seeded at the density of $5x10^5$, 24 h later cells were infected with rAd.E1^E4 (Davy et al., 2002) or rAd.β-galactosidase (Davy et al., 2002). Cells were harvested 24 h post infection and the pellets were resuspended in 150 µl/plate of NP40 lysis buffer (0.5 % (v/v) NP40/PBS/25 mM complete protease inhibitor cocktail) and then were incubated at 4 °C with rotation for 30 mins. The sample was subsequently pelleted by centrifugation (10,000 G, 15 mins, 4 °C). The supernatant was transferred to a 1.5 ml microcentrifuge tube and stored on ice. This was called the NP40 or soluble fraction.

The resultant pellet was resuspended in 100 µl/plate of 0.8 % (v/v) Empigen/PBS/25 mM complete protease inhibitor cocktail, using a 1 ml syringe fitted with a 23 gauge needle, the sample was then incubated at 4 °C and rotated for 30 mins. The extract was centrifuged and stored as before, this was called the empigen or keratin associated fraction. The remaining pellet was resuspended in 100 µl/plate of 9 M Urea/PBS and subsequently stored on ice, this formed the Urea fraction.

2.6.2.4 Trichloroacetic acid (TCA) Precipitation

Protein fractions from radioactively-labelled SiHa cells were used in a TCA precipitation to measure the amount of incorporated radiolabelled precursor. Briefly, 5 µl of sample was mixed with 5 µl of BSA (10 $mgml^{-1}$, NEB). The mixture was then applied to the centre of a 3 mm diameter glass fibre filter in a glass trough. A solution of cold 10 % (w/v) TCA was then added to the trough, which was incubated on ice for 30 mins.

The TCA was then drained from the trough and replaced with fresh 10 % (w/v) TCA, the trough was then incubated at R/T with agitation for 5 mins and this step was repeated once. The TCA was replaced with 95 % ethanol and the trough was incubated at R/T with agitation for a further 5 mins. Finally the filter was removed from the trough and left to air dry for 15 mins. The dry filter was transferred to a scintillation vial and 2 ml of Ready Safe™ scintillation fluid (Beckman) was added, the precipitated protein was then counted on a Beckman 6000SC scintillation counter.

2.6.2.5 Lambda protein phosphatase (λ-PPase) treatment of cell extracts

The empigen fraction of rAd.E1^E4 infected SiHa cells was used in λ-PPase experiments. Briefly, 15 µl of the empigen fraction was taken and incubated in 2 mM $MnCl_2$, 1 x λ-PPase buffer (NEB) for 1 h at 30 °C both with and without 400 U of λ-PPase (NEB). The samples were subsequently analysed by Western blotting.

2.6.2.6 GST pull-downs

Fractionated rAds infected SiHa cells or His-tagged bacterially expressed proteins were used in pull-down experiments with GST and GST fusion proteins bound to beads in 1 ml of pull-down buffer (1 % (v/v) NP40, 100 μgml^{-1} BSA/PBS, 25mM Complete protease inhibitor cocktail). In pull-downs performed with cell extracts the entire NP40 fraction and 10 % (v/v) of the empigen and urea fractions of 3 x 90 mm dishes of SiHa cells were used. To identify direct protein interactions His-tagged E1^E4 proteins (~0.5 µg) were used.

The mixtures were rotated at 4 °C for 1 h, and then the beads were pelleted by centrifugation (2000 G, 5 mins) and then resuspended in 1% (v/v) NP40/PBS and then repelleted by centrifugation (2000 G, 5 mins). This wash step was repeated 4 x with 1% (v/v) NP40/PBS. The liquid was removed by careful pipetting and the beads dried. Proteins were eluted from the beads in 20 µl of sample buffer (50 mM Tris.HCl, pH6.8, 100 mM DTT, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol) by boiling (5 mins, 95 °C). The beads were pelleted by centrifugation (10 000 G, 5 min), and the supernatant analysed by SDS-PAGE.

2.6.2.7 Co-immunoprecipitation

Protein G sepharose beads (300 µl) were equilibrated, in NP40 lysis buffer as described in section 2.6.1.2. Fractionated radioactively-labelled SiHa cells infected with rAds were diluted as described in section 2.6.2.6 and 50 µl of washed Protein G sepharose was added to each tube, the mixtures were rotated at 4 °C for 30 mins. The beads were then pelleted by centrifugation (5000 G, 5 mins), the resulting supernatants were transferred to fresh microcentrifuge tubes and 10 µl of anti-MBP16E1^E4 (Rabbit G) polyclonal (Doorbar et al., 1997) or rabbit serum (Sigma-Aldrich) was added to the mixtures which were rotated at 4 °C. After 30 mins, 50 µl of washed Protein G sepharose beads were added to each tube and they were rotated at 4 °C for 1 h. The mixtures were then washed and proteins were eluted, as described in section 2.6.2.6 and visualised by SDS-PAGE and autoradiography or Western blotting.

2.6.2.8 Preparation of proteins for SDS-PAGE

Protein extracts (~20 µg) or 10 µl of 50 % (v/v) slurries of GST fusion proteins bound to beads were mixed with sample buffer at the ratio of 2:1. Samples were then incubated at 95 °C for 5 mins and then chilled on ice. Samples were then placed at R/T for ~10 mins prior to electrophoresis.

2.6.2.9 Preparation of SDS-PAGE gels

SDS-PAGE gel solutions were prepared as shown below using Protean II mini-gel apparatus (Bio-Rad). In some cases pre-cast 4-12 % Bis-Tris gradient gels (Invitrogen) were used.

Components of SDS-PAGE gels:

SOLUTION	10 %	15 %	STACKING GEL
Water	4 ml	2.3 ml	6.8 ml
Protogel, 30 % (w/v) acrylamide: 0.8 % (w/v) bis-acrylamide (37.5:1)	3.3 ml	5.0 ml	1.7 ml
1.5 M Tris.HCl (pH 8.8)	2.5 ml	2.5 ml	--------
1.0 M Tris.HCl (pH 6.8)	--------	--------	1.25 ml
10 % (w/v) SDS	0.1 ml	0.1 ml	0.1 ml
10 % (w/v) APS (freshly prepared)	0.1 ml	0.1 ml	0.1 ml
TEMED	4 µl	4 µl	10 µl

2.6.2.10 SDS-PAGE electrophoresis

Samples were loaded onto the SDS-gels and separated by electrophoresis in SDS protein gel running buffer (25 mM Tris.HCl (pH 8.3), 200 mM glycine, 0.1 % (w/v) SDS), limiting to 150 V, 200 mA. The migration of Rainbow Molecular Weight (MW) Markers (Amersham Pharmacia Biotech) or Silver stain MW markers (Bio-Rad) through the gel were used to determine the size of separated proteins.

2.6.2.11 Western Blotting

Proteins were transferred to Immobilon membrane (Millipore, Watford, UK), by wet blotting on Mini Trans-blot (Bio-Rad) at 150 V, 200 mA for 2 h in transfer buffer (25 mM Tris.HCl (pH 8.3), 200 mM glycine, 20 % (v/v) MeOH). Blots were blocked for 2 h at R/T by agitating in blocking buffer (5 % (w/v) Marvel milk powder (Premier Brands UK, Ltd. The Wirral, UK) in PBS-T (0.1 % (v/v) Tween20 in PBS).

Primary antibodies were diluted in blocking buffer at the dilutions specified below and incubated with the blots at 4 °C, O/N. Blots were washed five times with PBS-T for 5 mins with agitation. The appropriate secondary polyclonal antibodies conjugated to

horse radish peroxidase (HRP) were diluted as specified below in blocking buffer, incubated with the blots at 4 °C for 1 h and washed as before.

Primary antibodies:

Target	Antibody	Dilution	Antibody type	Source
HPV16 E2	TVG261	1:1000	Purified mouse monoclonal antibody.	(Hibma et al., 1995)
HPV16 E1^E4	TVG402	1:20-1:100	Mouse monoclonal antibody, in tissue culture supernatant	(Doorbar et al., 1992)
HPV16 E1^E4	Anti-MBP16E1^E4 (Rabbit G)	1:2000	Rabbit polyclonal antiserum	(Doorbar et al., 1997)
HPV16 E1^E4	Anti-PETPAT	1:250	Anti-peptide, rabbit polyclonal antiserum	Figure 3.1
HPV16 E1	E1-N1	1:1000	Mouse monoclonal antibody, in tissue culture supernatant	(Masterson et al., 1998)
His-Tag (HHHHH)	His•Tag® monoclonal antibody	1:1000	Purified mouse monoclonal antibody	Novagen (Merck Biociences, Nottingham, UK)
α-tubulin	Anti-α-tubulin (T9026)	1:1000	Purified mouse monoclonal antibody	Sigma-Aldrich
Green fluorescent protein (GFP)	GFP (B-2): sc-9996	1:400	Purified mouse monoclonal antibody	Santa Cruz (Autogen Bioclear, Calne, UK)

Secondary antibodies:

TARGET	ANTIBODY	SPECIES	DILUTION	SOURCE
Mouse IgG	NA931	Sheep	1:2000	Amersham Pharmacia Biotech
Rabbit IgG	NA934	Donkey	1:1000	Amersham Pharmacia Biotech

Binding of secondary antibody was visualized by Enhanced Chemiluminescence (ECL), (Amersham Pharmacia Biotech), with blots being exposed to MXB X-ray film (X-ograph imaging systems, Tetbury, UK). Where specified fast DAB tablets (Sigma-Aldrich) were used to detect HRP activity, according to the manufacturer's protocol.

2.6.2.12 Coomassie staining of SDS-gels

SDS-gels were placed in Coomassie staining solution (0.125 % (w/v) Coomassie blue, 50 % (v/v) methanol, 10 % (v/v) acetic acid, 40 % (v/v) ddH_2O) for at least 1 h with

agitation. Subsequently, the gels were placed in destain solution (50 % (v/v) methanol, 10 % (v/v) acetic acid, 40 % (v/v) ddH_20) with a small foam bung and incubated O/N, with agitation.

2.6.2.13 Silver staining of SDS-gels

SDS-gels were fixed in 50 % (v/v) methanol, for 30 mins, prior to incubation in sodium thiosulphate ($Na_2S_2O_3$) solution (0.017% (w/v) $Na_2S_2O_3.5H_2O/ddH_2O$) for 1 min. The gels were then washed 3 x at 5 second intervals with ddH_2O. Next the gels were incubated with staining solution (1 % (v/v) formaldehyde (HCHO), 0.26 % (w/v) silver nitrate) for 8 mins and then washed three times at 5 second intervals with ddH_2O. Bands were visualised using developing solution (2 % (w/v) sodium carbonate, 0.042 % (v/v) HCHO, 0.004 % (w/v) $Na_2S_2O_3.5H_2O$). Over-development of protein bands was avoided by exchanging the developing solution with 1 % (v/v) acetic acid.

2.6.2.14 Detection of radioactive signal

Radioactive signals from ^{35}S labelled molecules were detected using a PhosphorImager (Storm860) (Amersham Pharmacia Biotech) and ImageQuant 5.0 software. Dried gels or labelled membranes were wrapped in saran wrap. Air bubbles were carefully removed by rolling over the saran wrap surface with a sawn off pipette and then the sealed gels or membranes were exposed to a light blanked phosphoImager plate typically O/N and if necessary longer exposures were performed. The exposed plates were then developed.

2.6.2.15 Band Densitometry

The net intensity of protein bands on X-ray film or on SDS-gels was estimated using Kodak digital science image station 440 CF and Kodak ID IS software.

2.6.3 Transcription assays

2.6.3.1 Sample preparation

C33a cells were seeded at the density of $2x10^5$ per each well of a 6-well dish, 24 h prior to calcium phosphate transfection using the Profection kit (Promega) as described in section 2.4.8. Cells were then lysed at 24 h, 48 h, 72 h or 96 h post transfection. Briefly, media was removed and then the adherent cells were washed with PBS before addition of 300 µl of reporter lysis buffer (Promega) to each well. Plates were swirled then incubated at 37 °C for ~15 mins. Cell lysates were transferred from each well into a separate 1.5 ml microcentrifuge tube and then vortexed for 10 secs prior to centrifugation (13000 G, 45 secs). All cells were co-transfected with hRL-TK (1 µg, Promega), which served as a transfection control (Appendix II).

2.6.3.2 Luciferase assays

Supernatants from lysed C33a cells were assayed for *Photinus* luciferase activity and also for *Renilla* luciferase activity using Luciferase Assay System and Renilla Assay System kits (Promega). Briefly, 100 µl of lysate was added to 100 µl of the appropriate luciferase substrate in an opaque 96-well dish immediately before luminescence measurements were taken on a Wallac Victor2 1420, multi-label counter (Perkin Elmer Life Sciences), using Wallac 1420 workstation software (Version 2.00).

Luciferase activity was determined by measuring the counts per second (CPS) with a 2 sec delay followed by a 10 sec read, the instrument measured counts from 0-1000000 CPS. A detergent compatible protein assay (Bio-Rad) was carried out according to the manufacturer's instructions so that CPS for the same number of cells with equivalent protein concentrations could be compared. Positive control transfections were performed in parallel with the pGL3.CONT (Promega) vector, which contains the SV40 promoter and enhancer driving expression of the *Photinus* luciferase gene, to establish the transfection efficiency on the day of the experiment (Appendix II).

The background level of the reporter vectors was determined by assaying the *Photinus* luciferase activity of cells transfected with the appropriate empty expression vector i.e. pMV11 or pIRES and the reporter vector itself i.e. pGL3.LCR.

The net *Photinus* luciferase activity for each sample was calculated by subtraction of the mean background activity from each sample. The assays were carried out in triplicate i.e. 3 independent transfections, which were repeated at least twice unless specified. To enable experiments performed on different days to be combined irrespective of differences in transfection efficiencies, net *Photinus* luciferase activity was converted to percentages, where the 100 % value was the highest value in a particular experiment.

2.6.4 Extraction and analysis of nucleic acids from cultured cells

2.6.4.1 Purification an digestion of plasmid DNA from cultured cells

Plasmid DNA was purified from transfected human cells using a commercial kit, Quantum Prep® Plasmid miniprep kit (Bio-Rad) according to the manufacturer's instruction with a few modifications. Briefly, 6 x 10^6 transfected C33a cells were harvested using 1 mM EDTA/PBS/2 % (v/v) trypsin-versene and pelleted (1250 G, 5 mins). Buffers supplied with the kit were used as specified for 2 ml of *E.coli* culture, with the exception that the neutralisation step of the alkaline-lysis protocol was extended for 16 h with rotation at 4 °C. Isolated DNA was digested by the addition of 5U of *Hind III* (NEB) or/and *Dpn I* (Stratagene) per sample and incubation at 37 °C for 3 h.

2.6.4.2 Purification of RNA from cultured cells

All tips used for RNA work was certified RNase and DNase free and all plastic and glass ware was treated with 0.1 % (v/v) DEPC/H_20. Briefly, 0.5ml microcentrifuge tubes were immersed in a 0.1 % (v/v) solution DEPC/H_20 and left to incubate at 37 °C O/N, prior to autoclaving and oven drying.

Both total RNA and messenger RNA (mRNA) species were isolated from cell culture using commercial kits. Total RNA was isolated using the RNeasy mini kit (QIAgen), using the manufacturer's protocol for isolation of total RNA from animal cells and mRNA was isolated using the Oligotex ® mRNA kit (QIAgen), using the manufacturer's protocol for isolation of PolyA$^+$ mRNA from animal cells.

2.6.4.3 Quantitation of RNA

RNA concentrations were assayed using a 1201 UV-visible spectrophotometer (Shimadzu, Milton Keynes, UK). RNA solutions were diluted in ddH_20 to 1:50-1:100 in a quartz cuvette and their absorbance at a wavelength of 260 nm measured (A_{260}). RNA concentrations were calculated assuming an A_{260} of 1 is equivalent to 0.04 $mgml^{-1}$.

2.6.4.4 DNase treatment of isolated RNA

mRNA samples (1μg) were treated with DNase I (Invitrogen) at R/T for 1 h in 20 μl reactions containing 1 x DNase I reaction buffer and 2 U of DNase I. Subsequently, DNase I was inactivated by the addition of 2.5mM EDTA solution to the reaction and incubation at 65 °C for 15 mins.

2.6.4.5 Complementary DNA (cDNA) synthesis

cDNA synthesis was carried out using the Cloned AMV First-Strand cDNA synthesis kit (Invitrogen) according to manufacturer's instructions, using 1 μg of DNase-treated mRNA as template and 2.5 μM of both oligo $(dT)_{20}$ and random hexamer primers/ 20 μl reaction. Samples were incubated on a Thermal Cycler 200 (GRI, Braintree, UK), according to the following parameters:

Step 1	25 °C	10 mins
Step 2	50 °C	45 mins
Step 3	85 °C	5 mins
Step 4	4 °C	hold

2.6.4.6 PCR reactions

PCR was used to amplify cDNA to confirm the presence of viral transcripts in cultured cells. The primers used were derived from previous studies and have been shown to be complementary to the target cDNAs produced from mRNA template sequences (Doorbar et al., 1990). PCR reactions were assembled using the following reagents:

Template	100 ng of cDNA
deoxy-nucleotides (dNTPs)	0.2 mM each
10 x PCR buffer (Novagen)	5 µl
$MgS0_4$, (Novagen)	1 mM
Forward primer	20 pmol
Reverse primer	20 pmol
KOD DNA polymerase (Novagen)	1 U
Total volume (made up with ddH_20)	50 µl

Samples were incubated on a Thermal Cycler 200 (GRI, Braintree, UK), according to the following parameters:

Step 1	94 °C	5 mins
Step 2	94 °C	30 secs
Step 3	50 °C	30 secs
Step 4	68 °C	30 secs
Step 5	Go to step 1 x 29 cycles	
Step 6	68 °C	10 mins
Step 7	4 °C hold	

PCR products and where specified RNA was analysed by agarose gel electrophoresis as described in section 2.3.2.3.

2.6.4.7 Quantitative PCR reactions

Quantitative PCR was used to amplify cDNA to measure the levels of specific mRNA transcripts in cell culture. Primer Express® software was used to design primer pairs with the following restrictions, the PCR product (amplicon) produced would be 50-150 bp in length, the primers would be between 9 and 40 bases in length, have a melting temperature (Tm) between 58 °C and 60 °C, the difference in Tm between the forward and reverse primer would be less than or equal to 2 °C, the GC content would be 20-80 % and a maximum of 2 G/Cs are present in the last 5 bases of the 3'-end of the primers. PCR reactions were assembled using the following reagents:

Template	20 ng of cDNA
Forward primer	20 pmol
Reverse primer	20 pmol
SYBR Green 2x mix (Applied Biosystems)	12.5µl
Total volume (made up with ddH_20)	20µl

Samples were incubated on an ABI® Prism 7000 sequence detection system (Applied Biosystems), according to the following parameters:

Step 1 50 °C 2 mins
Step 2 95 °C 10 mins
Step 3 95 °C 15 secs
Step 4 60 °C 1 min
Step 5 Go to step 1 x 40 cycles

2.6.4.8 Denaturing Gel Electrophoresis

RNA samples for northern blotting were separated on 1.2 % formaldehyde agarose (FA) gels buffered with 1 x FA buffer (200 mM MOPS (free acid), 50 mM NaAc, 10

mM EDTA-NaOH, pH 7), containing 1 μgml-1 EtBr. Samples were mixed with 5 x loading buffer (20 % (v/v) glycerol, 0.5 % (w/v) bromophenol blue, 4 mM EDTA, 2.7 % (v/v) formaldehyde, 3.2 % (v/v) formamide, 4 x FA buffer). Electrophoresis was carried out, at a constant voltage of 70 V, limiting to 200 mA. RNA bands were visualized by trans UV illumination and compared to RNA markers (Invitrogen) and to 28s and 18s rRNA. Digital images were captured with Kodak Image Station 440 and Digital Science 1D software (Perkin Elmer Life Sciences, Ltd., Cambridge, UK).

2.6.4.9 Preparation of agarose gels prior to transfer to nitrocellulose membranes

Non-denaturing agarose gels containing separated DNA were submerged in denaturing solution (1.5 M NaCl, 0.5 M NaOH) followed by neutralization solution (1.5 M NaCl, 1 mM EDTA, 0.5 M Tris-HCl, pH 7.2) both incubations were for 30 mins at R/T with agitatation. Both non-denaturing and denatured agarose gels were incubated in 10 x SSC for 30 mins with a buffer change after 15 mins to equilibrate the gels prior to transfer.

2.6.4.10 Transfer of Nucleic acids from agarose gels to nitrocellulose membranes

Hybond N+ membrane (Amersham Pharmacia Biotech) was pre-wetted with ddH_20 and then incubated for 15 mins in 10 x SSC, while transfer apparatus was assembled as shown in Figure 2.1. Briefly, 3 sheets of filter paper (wick) were laid over a plastic support frame, so the ends of the stacked filter paper sheets were dipped into the 10 x SSC filled tank. Next the equilibrated gel was inverted and placed onto the filter paper. Saran wrap was stretched around the gel to prevent contact between the filter paper wick and the stacked paper towels during capillary transfer. The membrane was then placed onto the gel surface and rolled with a sawn off pipette to remove air bubbles. Next a stack of paper towels ~12 cm high was placed on top the assembled apparatus followed by a weight of ~3 kg. The apparatus was secured and then left to transfer O/N.

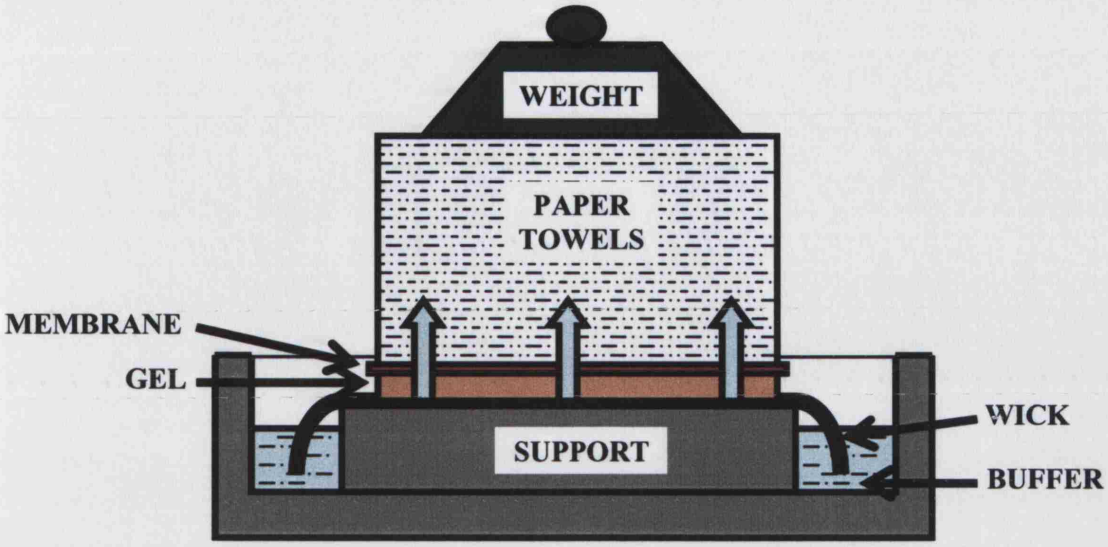


Figure 2.1 Diagram of the RNA and DNA transfer apparatus
The components of the apparatus are labelled, and blue arrows indicate the direction of capillary action.

2.6.4.11 Fixation of nucleic acids to a nitrocellulose membrane

Nucleic acids were fixed to a nitrocellulose membrane in a hybridisation tube, by baking the membrane for 2 h, at 80 °C in a hybridisation oven.

2.6.4.12 Pre-hybridisation of the membrane

The membrane in the hybridisation tube was incubated with 10 ml of pre-heated hybridisation buffer supplemented with 5 mg of denatured salmon sperm DNA (Sigma Aldrich) and left to rotate in a hybridisation oven at 65 °C for 1 h. Salmon sperm DNA was denatured prior to use by heating to 95 °C for 10 mins.

2.6.4.13 Radiolabelling of DNA probes

All radioactivity containing steps specified were carried out behind Perspex shielding and monitored using a Geiger counter. To produce the radiolabelled probe 50 ng of gel extracted DNA digest fragments were mixed with ddH_20 in a 0.5 ml microcentrifuge tube to give a final volume of 45 µl and heated to 95 °C for 2 mins and then rapidly cooled on ice. To the denatured DNA 5 µl of [α-^{32}P] dCTP (1.85 MBq) (Amersham Pharmacia Biotech) was added and then the combined solution was transferred to a tube containing a Ready-to-Go DNA labelling bead (Amersham Pharmacia Biotech) and was incubated at 37 °C for 30 mins. Radioactive labelling of

the probe was carried out on the same day as membrane hybridisation. Following the incubation 2 µl of 0.5 M EDTA was added to stop the labelling reaction.

2.6.4.14 Purification of radiolabelled probes

NICK™ columns (Amersham Pharmacia Biotech) were used to remove unincorporated nucleotides from the labelled probe. Briefly, excess liquid from the NICK™ column was removed and then 3 ml of TE buffer was added to the column, supported on a retort stand. Once all the TE buffer had drained through the column, the labelling reaction was added to the top of the column, followed by 400 µl of TE buffer. All of these column elutions were disguarded. Next a 1.5 ml microcentrifuge tube was placed below the column to which was added another 400 µl of TE buffer. This final elution contained the purified radiolabelled probe and was stored at 4 °C until used.

2.6.4.15 Hybridisation of the membrane

The purified labelled probe (200 µl) was heated to 95 °C for 5 mins then chilled on ice. This was then directly pipetted into the hybridisation buffer following prehybridisation, avoiding direct contact between the undiluted probe and the membrane. The membrane was then incubated with the diluted probe at 42 °C in a hybridisation oven with rotation O/N.

2.6.4.16 Removal of excess probe from the membrane

Wash solutions were prepared and pre-heated, prior to incubation with the membrane which was performed in a hybridisation oven with rotation at temperatures and for times as follows: 3 x (30 mins, 42 °C, 2 x SSC, 0.1 % (w/v) SDS)

2 x (40 mins, 55 °C, 0.2 x SSC, 0.1 % (w/v) SDS)

2.6.4.17 Detection of radioactive signal

Radioactive signals from ^{32}P labelled molecules were detected using a PhosphorImager as described in section 2.6.2.15.

2.6.5 Antibody Production

2.6.5.1 Activation of Keyhole Limpet hemocyanin (KLH)

Imject® mariculture lyophilised KLH (20 mg, Perbio Science) was reconstituted with 2 ml of degassed ddH_20 to give a concentration of 10 $mgml^{-1}$ in 0.083 M Na_2PO_4, 0.9 M NaCl. Water was degassed to displace dissolved oxygen, by passing a stream of nitrogen gas through it for 5-10 mins. The activating agent used with KLH was m-maleimidobenzoyl-N-hydroxysuccinimide (sulfo-MBS, Perbio Science); this was dissolved in conjugation buffer (0.083 M Na_2PO_4, 0.9 M NaCl, 1 M EDTA, pH 7.2) to give a concentration of 2 $mgml^{-1}$. The sulfo-MBS solution was added immediately to the KLH solution and the mixture was rotated at R/T for 1 h.

In the meantime, a PD-10 desalting column (Amersham Pharmacia Biotech) was equilibrated with 25 ml of conjugation buffer. Next the KLH-MBS solution was passed through the column and collected in 12, 0.5 ml elutions. A small amount of each fraction was taken and diluted 1 in 50 in conjugation buffer and then their OD_{280} was measured in a quartz cuvette, these measurements were used to produce a protein elution profile. The fractions that formed the peak were pooled together as they contained activated KLH molecules.

2.6.5.2 KLH-Peptide conjugation

The peptide sequence PETPATPLSC was synthesised by Peter Fletcher (NIMR) using the FastMoc solid phase peptide synthesis method, purified using reverse-phase high performance liquid chromatography (HPLC) and then freeze-dried (Tiburu et al., 2003). Purified peptide was dissolved in 1 ml of conjugation buffer. A 50 µl aliquot of unreacted peptide was removed and stored at 4 °C, for use later in a cysteine assay. The remaining peptide solution was added to the activated KLH and rotated at 4 °C, O/N. The percentage of coupled peptide produced was estimated the next day using a cysteine assay.

2.6.5.3 Cysteine Assay

A cysteine assay was performed as a measure of the total number of sulfhydryl groups, protein-bound sulfhydryl groups and free sulfhydryl groups present in the

coupling reaction. Briefly, cysteine standards were prepared by serial dilution of freshly reconstituted lyophilised cysteine in ddH_20, solutions ranged from 0.4 to 0.04 $mgml^{-1}$, 50 µl of each standard and of ddH_20 (blank) was taken and placed into individual glass test tubes. To each tube 100 µl of 1 $mgml^{-1}$ Ellman's reagent (5,5-Dithio-bis-2-nitrobenzoic acid (DTNB) dissolved in DTNB buffer (0.1 M Na_2P0_4, pH 8)) was added and 750 µl of DTNB buffer. The tubes were vortexed briefly and the absorbance of the solutions measured at an OD_{412}.

Unreacted peptide and conjugated peptide (50 µl) were diluted 1 in 3 with ddH_20 and 50 µl aliquots were reacted with Ellman's reagent as described, the values were multiplied by the dilution factor, 3 to give the concentration (μgml^{-1}) of the total sulfydryl groups and remaining free sulfydryl groups, respectively. The percentage of coupled peptide was calculated using the following equation:

$$\% \text{ Coupling} = \frac{[\text{Total sulfhydryl groups}] - [\text{Free sulfydryl groups}]}{[\text{Total sulfhydryl groups}]}$$

2.6.5.4 Dialysis

Conjugated KLH-peptide was dialysed into PBS. Briefly, samples were added to Slide-A-Lyzer® cassettes with a MW cut off of 10,000 (Perbio Sciences). The cassettes were then immersed in a 1 L glass beaker filled with cold PBS. The PBS was then stirred for 6 h with a metallic stirrer at 4 °C before the PBS was replaced with fresh PBS and stirred O/N at 4 °C. The dialysed proteins were then quantitated using a protein assay and diluted PBS to give a concentration of 400 μgml^{-1} in PBS, prior to immunisation.

2.6.5.5 Immunisation of rabbits

For polyclonal antibody production two New Zealand white rabbits were injected subcutaneously at 4 sites by Harlan Sera-Lab (Loughbourgh, UK), according to the following protocol.

DAY NO.	ACTION
0	Pre-bleed (5 ml) + Immunisation (0.5 ml of antigen and 0.5 ml of Freunds Complete Adjuvant)
14	Boost 1 (1 ml Freunds Incomplete Adjuvant)
28	Boost 1 (1 ml Freunds Incomplete Adjuvant)
35	Test bleed 1
42	Boost 1 (1 ml Freunds Incomplete Adjuvant)
49	Test bleed 2
56	Boost 1 (1 ml Freunds Incomplete Adjuvant)
63	Test bleed
70	Boost 1 (1 ml Freunds Incomplete Adjuvant)
77	Terminal bleed

2.6.6 Statistical Analysis

When given, the following the mean ($\overline{X}$), variance (sd^2), standard deviation (sd) and the standard error (SE) was calculated using the following formulas:

$$X = \frac{\Sigma x}{n} \qquad sd^2 = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}$$

$$sd = \sqrt{sd^2} \qquad SE = \frac{sd}{n-1}$$

Chapter 3 Identification of E1^E4 binding partners

3.1 Introduction

The levels, ratios and combinations of HPV and cellular proteins have been shown to change with epithelial differentiation (Apt et al., 1996; Ozbun and Meyers, 1998a; Ruesch et al., 1998; O'Connor et al., 2000; Weijzen et al., 2003). This is thought to affect the occurrence of functional interactions between HPV proteins, and between cellular and HPV proteins. Transcripts encoding 16E1^E4 have been isolated from undifferentiated HPV16 positive cells but E1^E4 protein has not been detected, suggesting that 16E1^E4 may be expressed in the basal layer of infected epithelium but at very low levels (Doorbar et al., 1990). In contrast, 16E1^E4 has been shown to be abundantly expressed in the upper layers of the epithelium, therefore E1^E4 is potentially expressed throughout infected epithelium at varying levels (Figure 3.1; (Doorbar et al., 1997)). Since it is possible that E1^E4 expression overlaps with the expression of all the viral and cellular proteins that are expressed during an HPV infection, the ability of E1^E4 to associate with a plethora of viral and cellular proteins was tested.

Co-immunoprecipations of E1^E4 complexes from epithelial cells transiently expressing E1^E4 were used to try and identify cellular binding proteins, and *in vitro* pull downs with glutathione S transferase (GST) viral fusion proteins and cell extracts containing E1^E4 were used to identify possible viral E1^E4 binding partners. The advantage of both these approaches is that they allow direct and indirect associations between E1^E4 and viral/cellular proteins to be identified.

3.2 Antibody choice for co-immunoprecipation

One of the most important determinants of successful co-immunoprecipation (co-IP) experiments is the antibody used. Polyclonal antibodies raised against a whole protein are commonly used for co-IPs, because they usually recognise several epitopes on the protein of interest. Alternatively, epitope specific-polyclonal antibodies or monoclonal

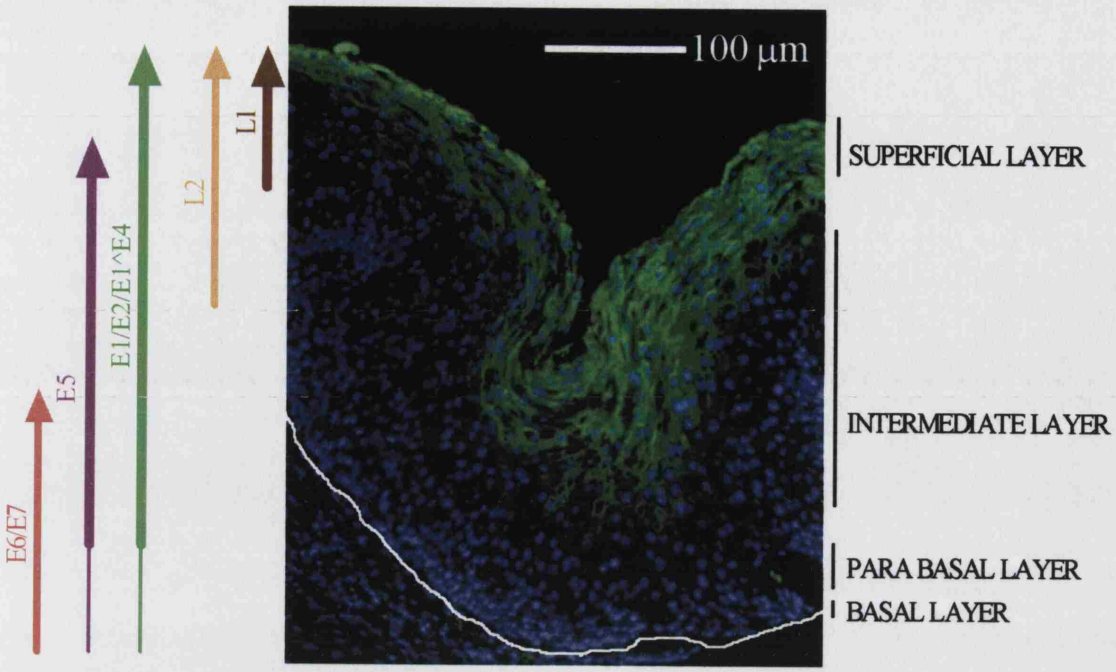


Figure 3.1 HPV16 E1^E4 expression in a CIN I lesion
E1^E4 is shown in green in the cross section, and was detected using an Alexa-488-TVG405 conjugate. The nuclei of cells were counterstained with DAPI, and are shown in blue. The solid white line marks the basal layer of the epithelium, and to the right of the cross section the positions of the different layers of the epithelium are approximated. To the left of the cross section, the putative expression patterns of HPV proteins in the different layers of the epithelium are indicated. The thickening of the arrow width represents an up regulation in protein expression.

antibodies can also be used. The disadvantage associated with using polyclonal antibodies for co-IPs is that unless they are affinity purified they frequently contain other non-specific antibodies, which could give artefactual results.

To increase the chances of identifying bone-fide cellular binding partners of E1^E4, it was decided to use a combination of epitope-specific antibodies in parallel with a polyclonal antibody raised against MBP-16E1^E4 (Doorbar et al., 2000). The latter antibody, and two other N-terminal epitope-specific antibodies were already available, however, there were none that specifically recognised sequences in the C-terminal portion of E1^E4 (Doorbar et al., 1992; Doorbar et al., 1997). For this reason it was decided to produce a C-terminal epitope-specific peptide antibody for the purpose of IP.

3.3 Peptide selection

Antibodies that recognise surface exposed epitopes are more likely to be useful for IP. The structure of E1^E4 has not yet been determined, and so surface exposed E1^E4 sequences can only be predicted. Hydrophilic sequences have the best chance of residing on the surface of a protein, and so a hydrophilicity algorithm was considered when choosing the peptide sequence to use as an antigen. Secondary structure prediction was also taken into consideration, as it is important to avoid sequences which are α-helical as short synthetic peptides do not usually form α-helices, this could limit the ability of the peptide to mimic an *in vivo* epitope (Figure 3.2).

Three suitable peptide sequences were identified as suitable candidates, i.e. residues 52-61, 63-71, and 74-84. The sequence that was chosen out of the three was the first one, as it met all the criteria that suggested it was a surface exposed epitope and in addition it had a cysteine residue at its C-terminus making it ideal for coupling to a carrier protein (Figure 3.3). Peptide coupling to large immunogenic carrier proteins is required to elicit an immune response to the peptide.

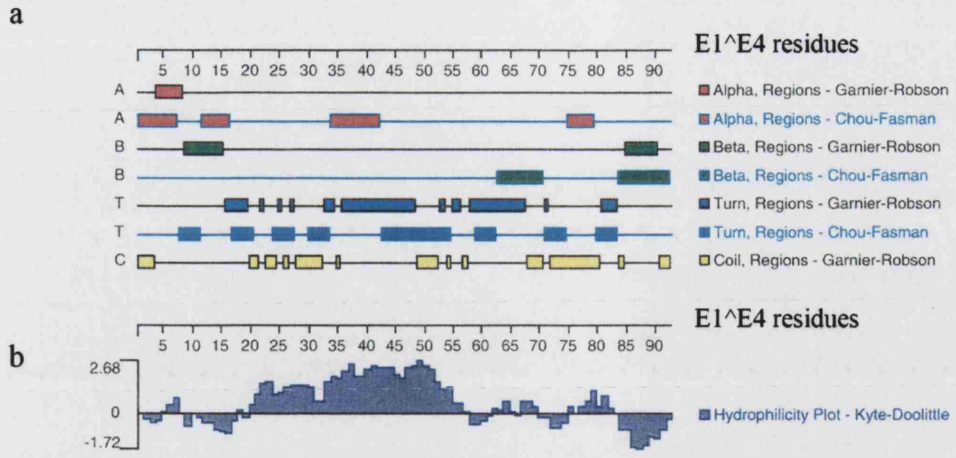


Figure 3.2 E1^E4 protein prediction
The methods used for each calculation are indicated, and the plots were generated using DNA Star software (Protean program module, Madison, USA). In both cases the x-axis corresponds to the amino acid sequence of E1^E4. (a) Colour coded secondary structure predictions for 16E1^E4, (b) Hydrophilicity plot produced by averaging the hydropathy values of 9 sequential amino acids. The y-axis corresponds to the hydropathy score (max value= +4.5 and min value= -4.5). Shaded regions above the line represent hydrophobic regions and the shaded regions below the line, represent hydrophilic regions.

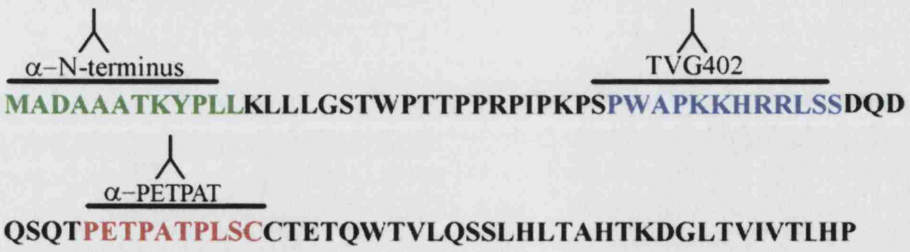


Figure 3.3 Epitopes of E1^E4 antibodies
The amino acid sequence of 16E1^E4 is shown. The green and blue highlighted sequences represent the epitopes recognised by the rabbit α-N-terminus peptide antibody, and the mouse monoclonal TVG402 antibody as labelled. The sequence shown in red is the peptide sequence that was used to produce the α-PETPAT antibody.

3.4 Peptide coupling to keyhole limpet hemocyanin (KLH)

KLH isolated from the mollusc *Megathura creulata*, was used as a peptide carrier protein, following activation by m-maleimidobenzoyl-N-hydroxysuccinimide (sulfo-MBS). Activated KLH was separated from excess reagents and the succinimide coupling bi-product by gel filtration, former to peptide conjugation. Sulfo-MBS covalently cross-links exposed primary amines of KLH lysine side-chains to free sulfhydryl groups of the terminal cysteine residue of the peptide. Coupling efficiency was determined using a cysteine assay, and was found to be 27 %. Efficiencies of 21-90 % have been shown to be useful immunogens for the generation of E1^E4 specific antibodies (Peh, 2002).

3.5 Generation of an E1^E4 specific antibody

The peptide conjugate was used to immunise two New Zealand white rabbits (Harlan Sera-Lab, Loughbourgh, UK). Blood samples were taken from the rabbits prior to immunisation (pre-bleeds), and also at 49 and 63 days following immunisation (test bleeds). The pre- and test bleeds were used for Western blotting of MBP and MBPE1^E4 (Figure 3.4). The pre-bleed did not react with either MBP or MBPE1^E4 at the concentration tested. Specific immunogenic responses of the test bleeds against MBPE1^E4, but not against MBP alone was seen, which appeared to be stronger in the third-bleed compared to the second-bleed. The responses to immunisation were found to be comparable in both rabbits, and so terminal bleeds were taken and the rabbits were sacrificed.

3.6 Characterisation of 16E1^E4 specific antibodies

TVG402, α-MBPE1^E4 and α-PETPAT antibodies were tested for reactivity against differentially phosphorylated forms of full length 16E1^E4 protein (Figure 3.5). Two E1^E4 bands that migrate at ~13K and 10K following separation on a 15 % polyacrylamide gel are present in E1^E4 containing cell extract. Treatment of E1^E4 samples with lambda-protein phosphatase (λPPase), an enzyme that can remove

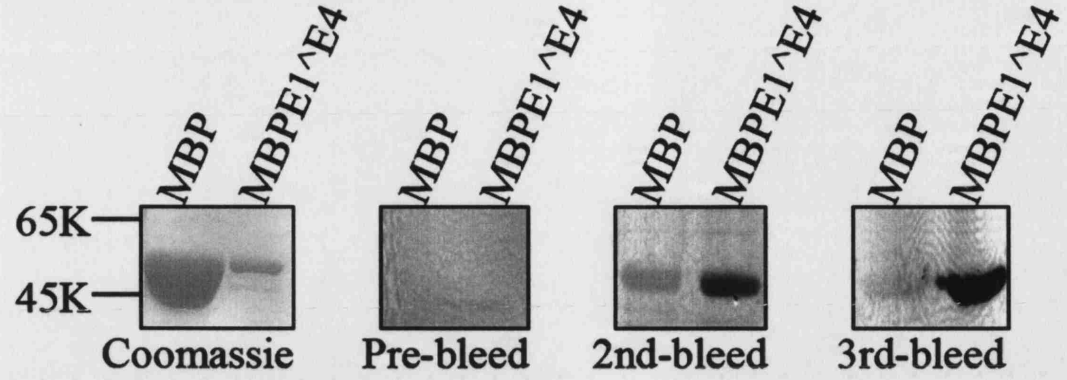


Figure 3.4 Specificity testing of the α-PETPAT antibody
Coomassie stained SDS-polyacrylamide gel (10 %) and Western blots of MBP and MBPE1^E4. Pre-, 2nd and 3rd bleeds of a rabbit immunised with KLH-PETPAT were used for Western blotting at a dilution of 1:250.

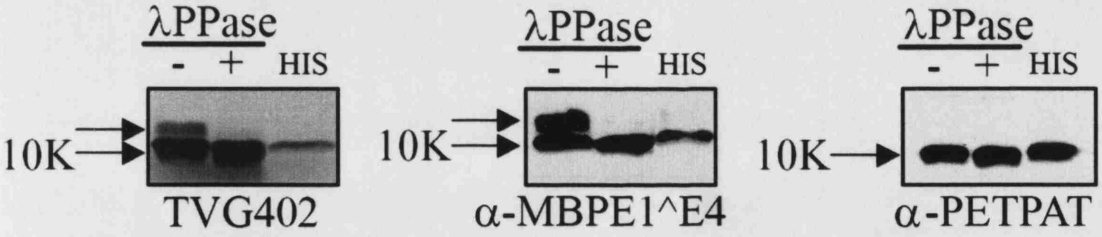


Figure 3.5 Identification of differences in specificity of E1^E4 antibodies
Western blots with E1^E4 specific antibodies as labelled of E1^E4 containing cell extract with (+) and without (-) lambda phosphatases treatment (λPPase), and of bacterially expressed His-E1^E4 (HIS), prepared by Dr. Pauline McIntosh (NIMR).

phosphates from serine, threonine, and tyrosine residues, resulted in the loss of the ~13K migratory form of E1^E4. This confirmed that the ~3K shift in migratory size of E1^E4 from its predicted size (10K) was due to phosphorylation (Wang et al., 2004). Both the unphosphorylated 10K (or unphosphorylated form of E1^E4, as it will be referred to) and 13K (phosphorylated) forms of E1^E4 were recognised by TVG402 and α-MBPE1^E4 by Western blotting, but only the unphosphorylated form was recognised by the α-PETPAT antibody.

In addition to the phosphorylated and unphosphorylated E1^E4, a third form of E1^E4 was sometimes seen following Western blotting with TVG402 of His-E1^E4 samples extracted from bacteria and of SiHa cell extract containing high levels of E1^E4 expressed from the recombinant adenovirus, rAd.E1^E4. This ~8K form of E1^E4, like phosphorylated E1^E4, was only found in the keratin-associated, and insoluble fractions of the cell. The N-terminal antibody did not recognise this form of E1^E4, suggesting it lacks at least the first 11 amino acids of the E1^E4 protein (Figure 3.6).

3.7 Co-IP with anti-MBPE1^E4

SiHa cells transiently expressing E1^E4 from rAd.E1^E4 were metabolically labelled with ^{35}S-methionine and ^{35}S-cysteine before sequential fractionation with NP40 (N), empigen (E), and urea (U) buffers. IPs with an antibody raised against MBPE1^E4 were performed with these fractionated cell extracts, or with rabbit serum from a non-immunised rabbit. The proteins eluted from each IP was divided into two, one sample was Western blotted for E1^E4 and the other was exposed to a phosphorimager cassette following SDS-PAGE.

Western blotting showed that unphosphorylated E1^E4 was immunoprecipitated from the NP40 fraction, and both phosphorylated and unphosphorylated forms of E1^E4 were immunoprecipitated from the empigen and urea fractions with an E1^E4-specific antibody, but not with rabbit serum (Figure 3.7a and b). Immunoprecipitated E1^E4 could not be detected by autoradiography, as it contains only two cysteine residues and possibly one methionine residue (as maybe cleaved off). However, nine radioactively

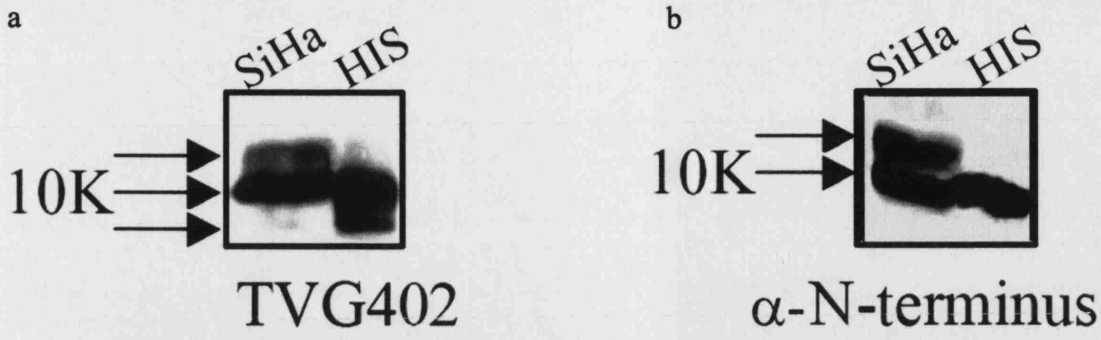


Figure 3.6 Detection of an N-terminally cleaved form of 16E1^E4
(a) Western blot with the TVG402 antibody of the empigen fraction of SiHa cells containing E1^E4 (SiHa) and bacterially expressed His-E1^E4 (HIS), prepared by Dr. Pauline McIntosh (NIMR). Both samples contain the 8K form of E1^E4. (b) Western blot with the α-N-terminus antibody of the same samples shown in part a.

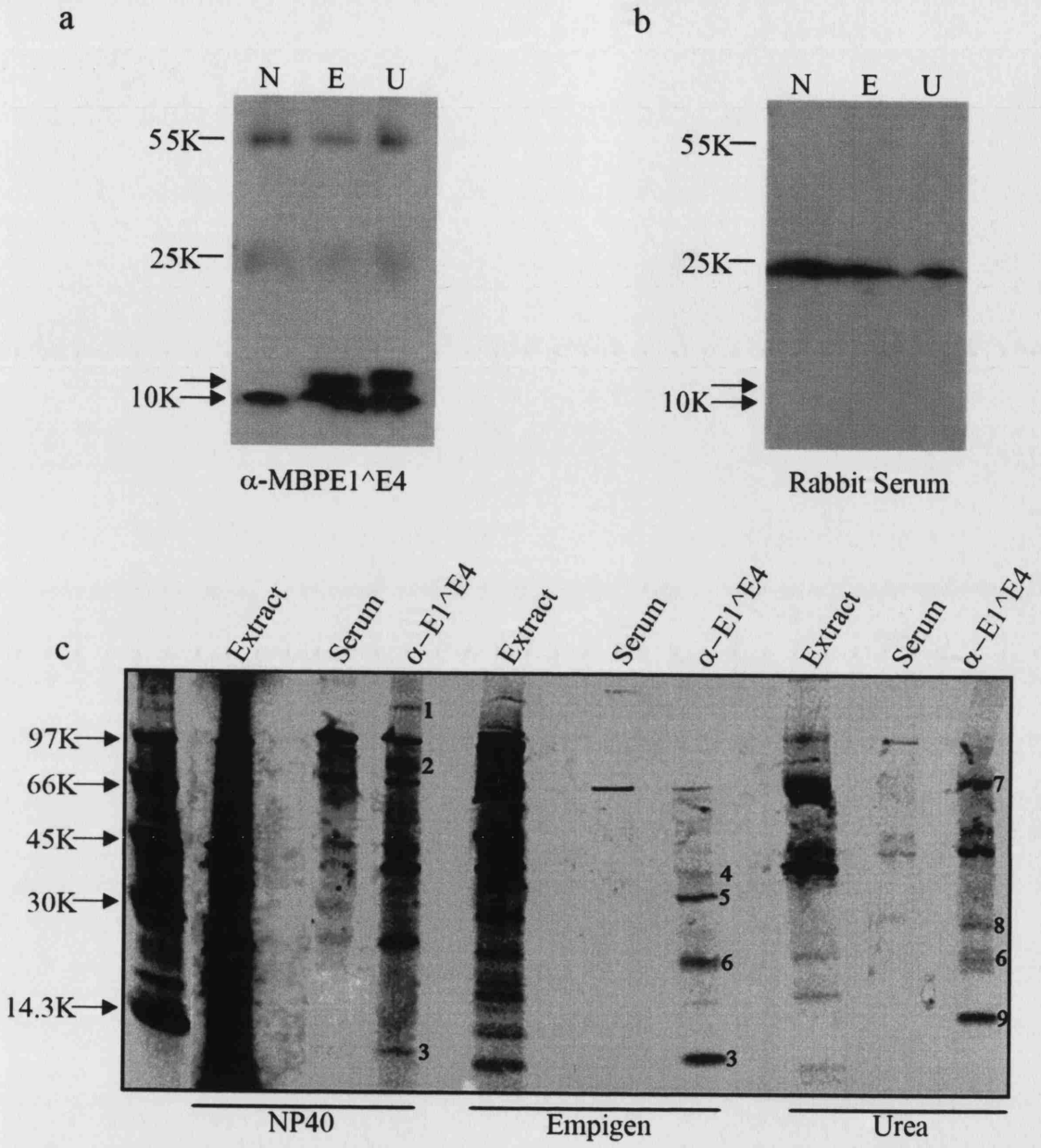


Figure 3.7 E1^E4 co-immunoprecipation
Radioactively labelled SiHa cells containing E1^E4, were divided into three detergent soluble fractions, i.e.NP40 (N), empigen (E) and urea (U), which were used in co-immunoprecipation reactions with rabbit α-MBPE1^E4 or with serum from a non-immunised rabbit. (a) and (b) Anti-E1^E4 (TVG402), Western blots of co-immunoprecipation reactions. (c) Autoradiograph of co-immunoprecipation reactions.

labelled proteins were detected in the E1^E4 specific co-IPs, which were not present in the control IPs (Figure 3.7c). Protein markers were used to estimate the sizes of co-immunoprecipitated proteins.

Band sizes and possible cellular candidates are summarised below.

NP40	Empigen	Urea	Suspects	Reference
112K(1)				
77K(2)				
		69K(7)	E4-DBP (E1^E4 DEAD box binding protein)	Yeast 2-hybrid screen (Doorbar et al., 2000)
	41K(4)			
	35K(5)			
		28K(8)	14-3-3	Yeast 2-hybrid screen (Dr. John Doorbar (NIMR), *personal communication*)
	21K(6)	21K(6)	Pin-1	16E1^E4 associates strongly with an *E.coli* peptidyl prolyl transisomerase (Dr. Pauline McIntosh, NIMR, Personal communication)
		15K(9)		
12K(3)	12K(3)		FKBP12	16E1^E4 associates with FKBP12 protein in *E.coli* (Dr. Pauline McIntosh, NIMR, Personal communication)

The next step was to identify the cellular candidates that were co-immunoprecipatated with E1^E4 using α-MBPE1^E4. This required the co-IP to be performed on a large scale, so that proteins were immunoprecipitated at levels that allowed them to be detected by less sensitive methods such as Coomassie blue staining of polyacrylamide gels, so that they could subsequently be extracted from the gel, and identified by matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy. Although the co-IP conditions had been optimised using α-MBPE1^E4 on a small scale, several problems became apparent during the bulking up procedure. Firstly, the detergent concentrations and buffer volumes used for fractionation and for IP could not be extrapolated linearly with the same results, and thus needed to be reoptimised. Secondly, increasing the amount of antibody used in the experiments to enable more proteins to be co-immunoprecipitated caused distortion of migration patterns of

proteins by SDS-PAGE, due to the high levels of antibody heavy and light chains that entered the gel.

Antibody-Protein G sepharose cross-linking, and varying of the amount of cell extract, blocking agent, and detergents, used for IPs were examined in turn and some progress was made in solving the problems identified but the technique still required further optimisation. However, the initial small scale IPs, had lead to the identification of a suitable fractionation procedure of SiHa cells transiently expressing E1^E4, and also conditions that preserved E1^E4-protein interactions. It seemed logical therefore, to use these same conditions to examine whether other HPV proteins could associate with E1^E4, as this would allow the interaction of different phosphorylated forms of E1^E4 to be assessed.

3.8 Expression of GST fusion proteins

HPV proteins that were to be tested for E1^E4 binding in pull-down assays, were expressed as GST fusions in bacteria. The production and purification of GST, GSTE2, GSTE7 and GSTL1 with minimal degradation and contamination with other bacterial proteins was achieved without much deviation from standard preparation conditions, outlined in sections 2.6.1.4 and 2.6.1.6. Although, on occasions where a contaminating 60K band was detected in GSTE2 preparations, additional wash steps were performed with ATP (5 mM) supplemented wash buffer. This band was suspected to be the bacterial chaperone protein, GroEL, which has been shown to co-purify with GSTE2, and was effectively removed by additional washing steps (Thain et al., 1997). The isolation procedures for GSTE1, GSTE1^E4, GSTE5, GSTE6 and GSTL2 were not as straight forward, and required significant adaptation of the standard protocol.

The purification of GSTE1 and GSTE6, have been shown previously, and so the conditions used by these authors were tried (Hibma et al., 1995; Elston et al., 1998). GSTE1 culture medium supplemented with betaine and sorbitol and IPTG induction at a lower temperature (25 °C instead of 30 °C) but for longer (overnight (O/N) instead of 2 h) was tried as these measures are thought to reduce the formation of protein aggregation. This method did slightly increase the amount of soluble GSTE1 that was

extractable using NP40 lysis of bacteria but the yield was still low. To increase the amount of full length protein produced, the culture was induced at 27 °C, for 6 h, and to increase the solubility of GSTE1, the ionic detergent sarkosyl was used instead of the non-ionic detergent NP40 (Figure 3.8a; (Frangioni and Neel, 1993)). Sarkosyl was also used for the extraction of full length GSTE5 from bacteria.

The supplementation of the GSTE6 growth media with betaine and sorbitol helped produce full length GSTE6, although there still appeared to be high levels of degradation (reflected by the protein ladder seen between full length protein (43K) and the GST-sized protein (26K) and a 66K contaminant was also co-purifying with GSTE6. It was found that by decreasing the concentration of IPTG used for induction the level of degradation in GSTE6 preparations could be reduced, and increasing the length of the washing steps (from 2 to 15 mins) eliminated the 66K contaminant from the preparations (Figure 3.8b). The use of low concentrations of IPTG means that less T7 RNA polymerase is produced by the IPTG-inducible lacUV5 promoter, this in turn decreases the transcription of GSTE6, which is under the control the bacteriophage T7 promoter ultimately decreasing the level of GSTE6 protein expression to levels that can be tolerated by the host bacteria.

GSTE1^E4 was poorly expressed under standard conditions, and initially it was difficult to even detect the presence of a degradation product, it was not immediately clear why this was i.e. was the protein was poorly expressed, rapidly degraded, or insoluble. In an attempt to detect any trace of E1^E4, cultures were induced using saturating concentrations of IPTG for a short time (1 mM IPTG, for 2 h) to produce high levels of E1^E4. This experiment showed that full length E1^E4 was expressed but was rapidly degraded, judging from the strong 26K band

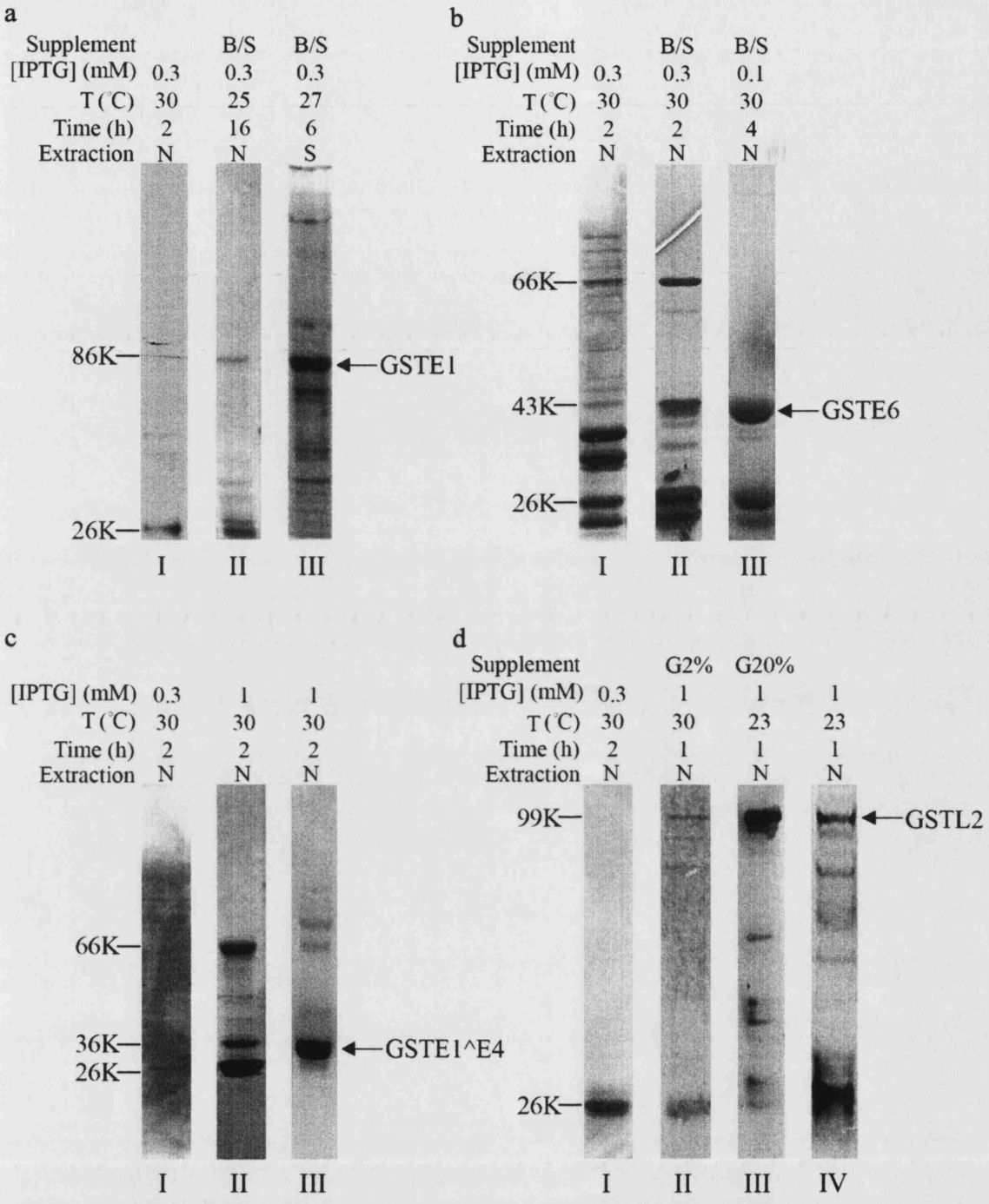


Figure 3.8 Optimisation of GST fusion protein preparations

Coomassie blue stained GST fusion proteins separated by SDS-PAGE. Above each lane summary of the growth (media supplementation i.e. 2.5 mM betaine and 0.5 M sorbitol (B/S), or percentage (w/v) glucose (G)), induction (concentration of IPTG ([IPTG]) used, temperature (T) and duration of induction) and extraction conditions (NP40 lysis (N), sarkosyl lysis (S)) used are summarised. Additional modifications to the standard protocol (Section 2.6.1.6) are specified where appropriate. The position and sizes of full length fusion proteins are labelled. The sizes of major protein contaminants and degradation products are also indicated. (a) GSTE1 preparations. (b) GSTE6 preparations. To increase the purity of the GSTE6 preparation (III) 5-6 additional Net-N wash steps were introduced. (c) GSTE1^E4 preparations. To be able to produce high levels of GSTE1^E4 in preparation (III), the pGEX.16E1^E4 construct was retransformed into BL21(DE3) star cells. (d) GSTL2 preparations. To increase the sensitivity of protein detection in the GSTL2 preparation (III) the SDS-polyacrylamide gel was silver stained. The pGEX.16L2 construct was retransformed into BL21(DE3) pLysS cells to be able to produce Coomassie blue detectable levels of GSTL2 in preparation (IV).

seen in lane 2 of Figure 3.8c. Several attempts were made to optimise growth conditions as outlined for GSTE1, and to reduce degradation, but the degraded band was consistently found to be equal or stronger than the full length fusion protein in intensity. To see whether changing the bacterial clone used would affect protein expression the pGEX.16E1^E4 construct was retransformed into BL21(DE3) star cells, which were used to prepare GSTE1^E4. It was found using this clone that full length E1^E4 protein could be produced with little or no degradation, with little deviation from the standard protocol (Figure 3.8c).

The major difficulty associated with GSTL2 production was that when grown in liquid culture, significant levels of bacterial cell death occurred, suggesting that low levels of GSTL2 expressed as a result of leaky transcription from the T7 promoter was toxic to the cells. In an attempt to overcome this problem, the growth medium was supplemented with 2 % (w/v) glucose. Glucose can repress the lacUV5 promoter reducing leaky expression of the T7 RNA polymerase, and thus the transcription of GSTL2 which is under the control of the T7 promoter. Glucose was excluded from the growth medium prior to induction with saturating IPTG concentrations for 1 h, to maximise transient GSTL2 expression, but this did not significantly improve GSTL2 production as cell death was still apparent in the culture.

To increase the repressive effect of glucose, its concentration in the growth media was increased from 2 to 20 % (w/v) prior to induction, which resulted in the production of full length GSTL2. However, the levels of GSTL2 produced were still too low to be seen by Commassie blue staining i.e. <0.1 μg of GSTL2 was present in 10 % of the preparation. Useful quantities of GSTL2 i.e. ≥5 μg were only produced following the transformation of pGEX.16L2 into BL21(DE3) pLysS *E.coli*, although high levels of protein degradation were still evident in the preparation (Figure 3.8d). The bacteria BL21(DE3) pLysS harbours a plasmid that encodes T7 lysozyme, which can bind to the T7 promoter and repress its activity, inhibiting the transcription of downstream genes in IPTG-free culture medium.

All the fusion proteins were finally produced at levels that could easily be detected by Coomassie blue staining of 4 % of bacterial preparations. Minimal degradation and only minor levels of chaperone proteins were detected in all the GST fusion protein

preparations. However, the yield of full length protein varied significantly between different protein preparations (Figure 3.9). For this reason densitometry of Coomassie stained full length protein bands eluted from glutathione-coated sepharose beads was performed to roughly estimate the amount in µl, of GST fusion protein bound to beads that were equivalent to ~5 µg. It was assumed that the Coomassie blue dye stained all the GST fusion proteins equally as all the proteins were fused to GST. The quantification of the amount of GSTL2, used in each pull-down was made difficult by the high levels of protein degradation that were apparent in the preparations, as potentially degraded GSTL2 could participate in complex formation with E1^E4 in addition to full length protein.

3.9 GST fusion protein pull-downs with E1^E4 containing cell extract

Detergent fractionated E1^E4-containing SiHa cell extracts were used in pull-down experiments with equivalent concentrations of GST and GST fusion proteins. The GSTE1^E4 pull-down served as a positive control, because E1^E4 self-association has been previously been shown (Wang et al., 2004). No association was seen when pull-downs were carried out with the negative control, GST, or with fusion proteins containing E1, E5 or E6.

The fusion proteins containing E2, E7, L1 and L2, as well as E1^E4 itself, did however show binding to E1^E4. GSTE2 and GSTL1, associated with both the phosphorylated and unphosphorylated forms of the protein. GSTE1^E4, GSTE7 and GSTL2 showed preferential binding to the unphosphorylated form of E1^E4, although on longer exposure of the Western blot, an association between the phosphorylated form of E1^E4 and L2 could be detected (Figure 3.10).

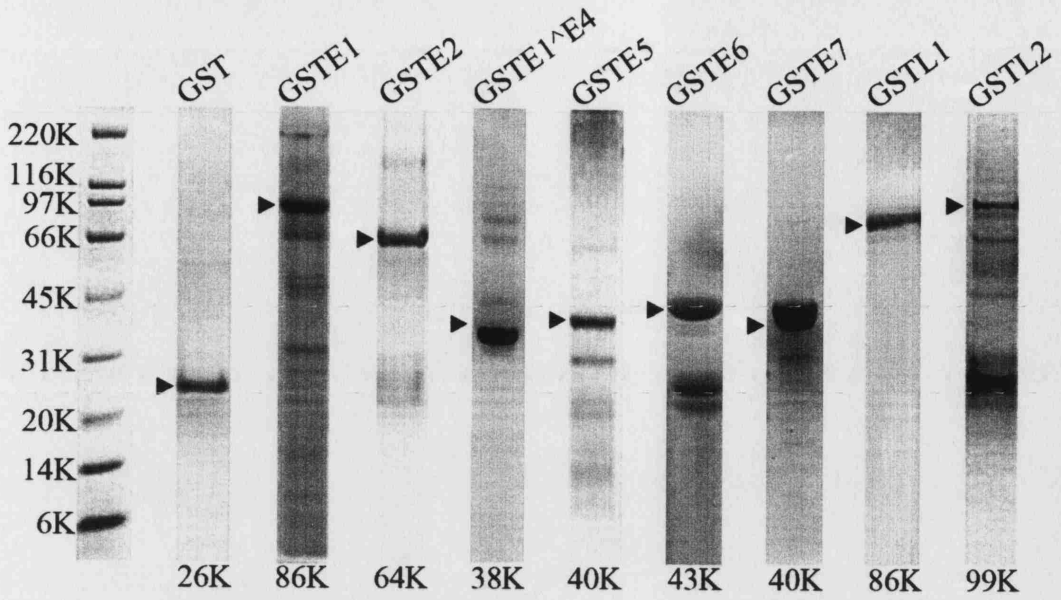


Figure 3.9 Purified GST/GST viral fusion proteins
GST and GST fusion proteins (4 % of preparations) separated by SDS-PAGE on 4-12 % Bis-Tris gels and stained with Coomassie blue. The full length fusion proteins are indicated by arrows, and the sizes of the fusion proteins can be estimated from the molecular mass markers in the left lane but are also specified below each lane.

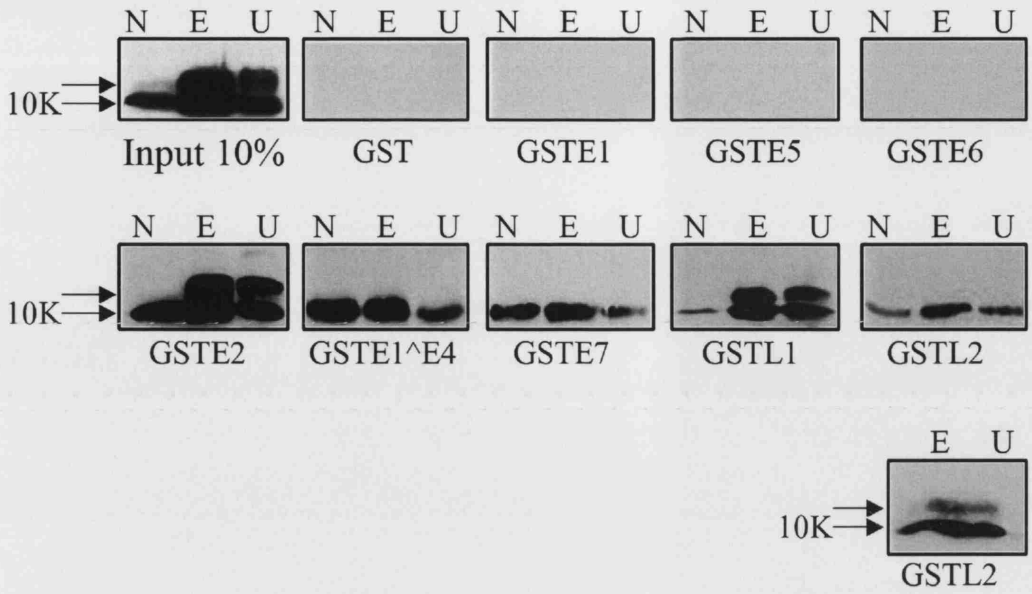


Figure 3.10 GST/ GST fusion protein pull-downs with E1^E4
A series of E1^E4 Western blots with the TVG402 antibody, of the E1^E4 containing NP40 (N), empigen (E) and urea (U) fractions of SiHa cells, used for pull-downs or eluted from GST, and GST fusion proteins following pull-downs. The panels are labelled with the GST/GST fusion proteins (~5 μg) that were used for each set of pull downs, and the lanes within each panel are labelled with the cell fractions that were used for the pull downs. The lower GSTL2 panel shows an over-exposure of a Western blot of E1^E4 protein eluted from GSTL2 following empigen (E) and urea (U) pull downs.

These GST pull-down experiments with E1^E4, show that unphosphorylated E2, E7, L1 and L2 can form a complex with E1^E4. However, it is not clear from these experiments if the associations with E1^E4 are direct or mediated by other proteins present in the cell extract. To answer this question pull-downs were repeated, but with purified Histidine-tagged 16E1^E4 (His-E1^E4) extracted from bacteria.

3.10 GST pull-downs with His-E1^E4

His-E1^E4 was purified to homogeneity from *E.coli*, using cobalt-based metal affinity resin under denaturing conditions and was subsequently refolded and dialysed into Tris buffer (by Dr. Pauline McIntosh, NIMR, Figure 3.11a). E1^E4 (~0.3 μg/pull-down), was promptly mixed with GST or with GST fusion proteins that had been shown to form a complex with E1^E4 from cell extracts. All the GST fusion proteins bound to His-E1^E4, however a weak association between E1^E4 and GST alone was also detected (Figure 3.11b). In these pull-downs the ratio of E1^E4 to GST/GST fusion proteins was high, compared to those performed with cell extract. It was thought that this could have increased the chances of a non-specific interaction occurring between GST and E1^E4 (Figure 3.11b). For this reason the pull-downs were repeated with the same preparation of refolded E1^E4 but with less GST/GST fusion proteins (~2 μg) per a pull-down.

The results were unexpected, strong bands of monomeric E1^E4 were detected in all the pull-downs, including the pull-down with GST. In addition, other E1^E4 antibody-reactive bands, were found to be present at ~28K and ~38K in all lanes, with additional bands being occasionally apparent. In the GSTE1^E4 pull-down, some of the additional bands (38K, 30K and 28K) correspond in size to GSTE1^E4 and its degradation products. The extra bands that were not monomeric E1^E4 or related to GSTE1^E4, but were detected using an E1^E4 specific antibody on the Western blot are thought to be E1^E4 multimers (Figure 3.11c).

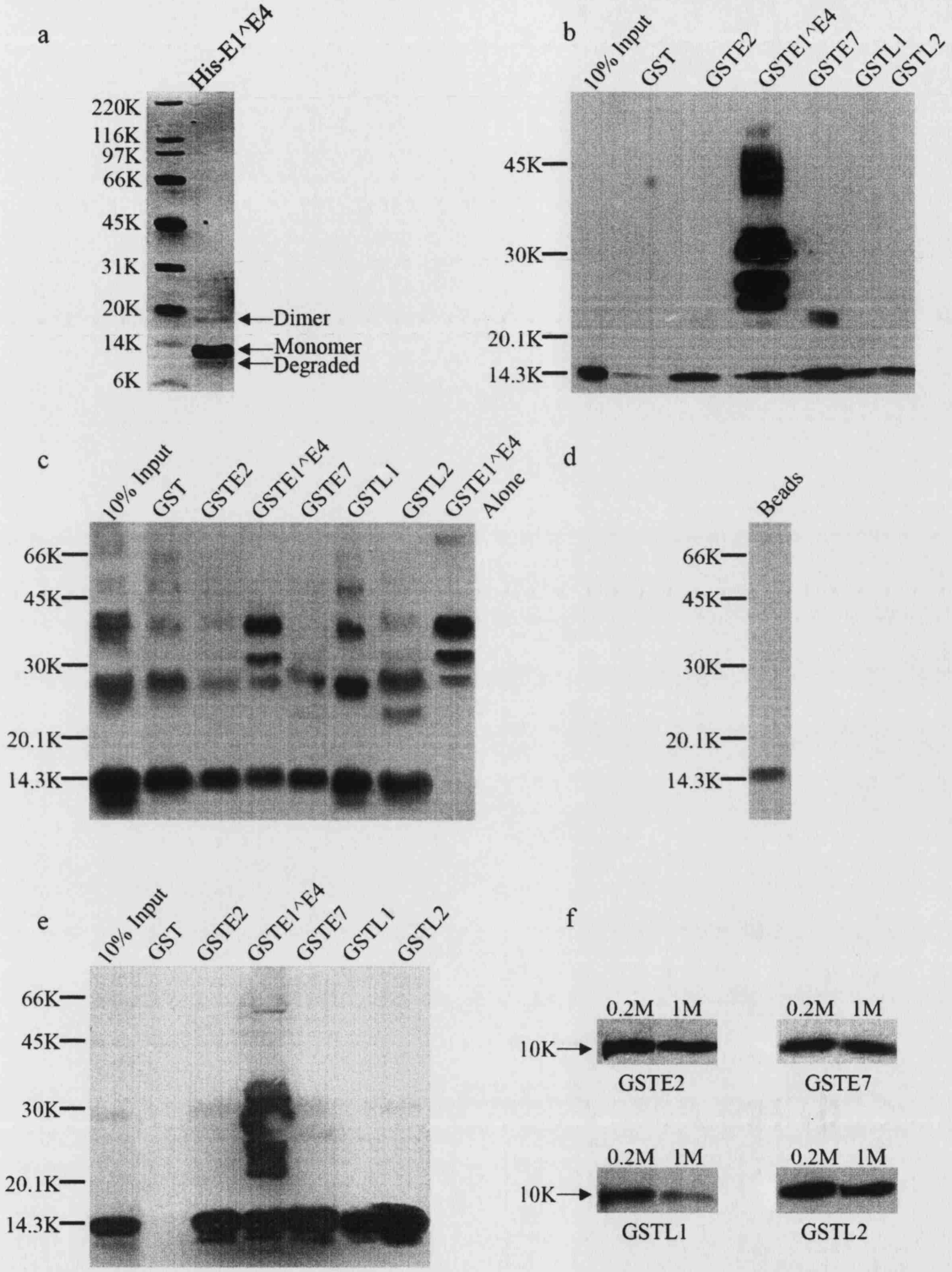


Figure 3.11 Direct binding of E1^E4 with HPV proteins

(a) Purified His-E1^E4 separated by SDS-PAGE and silver-stained. The different forms of E1^E4 present in the sample are labelled. The remaining panels are Western blots with TVG402 of His-E1^E4 pull-downs. (b) Freshly refolded E1^E4 protein (~0.5 μg) was used in the pull-downs with GST proteins (~5μg). (c) Refolded E1^E4 protein was stored at 4 °C for 2 weeks, prior to use in these pull-downs with GST proteins (~2 μg). In the last lane of the Western blot, GSTE1^E4 alone is shown. (d) E1^E4 protein in urea buffer was used for these pull-downs with GST proteins (~5μg). (e) Pull-down was performed as in d, but with glutathione-coated beads only. (f) E1^E4 protein in urea buffer was used for these pull-downs performed in the presence of low (0.2 M), or high (1 M) NaCl concentrations.

It was thought that the apparent non-specific association of monomeric E1^E4 and E1^E4 mulitmers with GST could be due to E1^E4 precipitation following refolding. To test this, a pull-down was performed with small quantities of glutathione-coated beads (2 µl) alone, and as suspected an E1^E4 precipitate was detected following Western blotting (Figure 3.11d). To circumvent this problem, His-E1^E4 was used in pull-down experiments without refolding. Urea-extracted His-E1^E4 was found to associate with GSTE2, GSTE7, GSTL1, GSTL2, and GSTE1^E4, but did not with GST alone. All the GST-HPV fusion proteins also bound to the N-terminally cleaved form of E1^E4 (8K) in these assays, suggesting that at least the first 11 amino acids of the E1^E4 N-terminus are not involved in mediating interactions with other HPV proteins (Figure 3.11e).

The HPV-E1^E4 protein interactions tested in this experiment were not disrupted by urea, a compound which is known to disrupt hydrophobic interactions. Urea competes with hydrophobic protein interactions by H-bonding with amino acid functional groups, which are mediating the association (Bennion and Daggett, 2003). This suggests the interactions between E1^E4 and E2, E7, E1^E4, L1 and L2, may be either strongly hydrophobic or ionic in nature. To test if the interactions between novel E1^E4 binding partners and E1^E4 were ionic, pull-downs were repeated as before but in the presence of high salt concentrations. High salt specifically reduces electrostatic (i.e. ionic) interactions, by increasing the amount of ions in solution, these tend to "insulate" charged protein side-chains, without significantly affecting hydrophobic interactions. All the HPV proteins tested still bound to E1^E4 in the presence of high salt. Although slight decreases in the amount of E1^E4 that was associated with E2, E7 and L2 could be seen, there were no significant differences in binding, except to L1 (Figure 3.11f). This suggests that hydrophobic interactions contribute to the binding of E1^E4 to all four HPV proteins, but that electrostatic forces appear to play a significant role in mediating the L1-E1^E4 association.

3.11 Validation of the E2-E1^E4 interaction

E2 and E1^E4 are transcribed together from the early promoter in the lower layers of the epithelium, and are upregulated during the productive stage of the life cycle

following activation of the differentiation-dependent promoter. In addition, both proteins have previously been shown to play cooperative roles in genome amplification and possibly also in virion egress. This suggests that the E2 and E1^E4 proteins may be functionally linked, like many other HPV proteins with overlapping ORFs, and for these reasons it was decided to focus on the E2/E1^E4 association.

Although the E2/E1^E4 association has been shown in previous experiments, to ensure the interaction with E1^E4 was not due to the presentation of E2 as a GST fusion protein, the reverse pull-down experiment was performed i.e. GSTE1^E4 was used in pull-downs with E2 containing cell extract. To enable high levels of E2 expression and extraction, E2 was produced in MCF-7 cells and extracted using RIPA buffer (Penrose and McBride, 2000; Bellanger et al., 2001). In parallel the binding of E2 to GST, GSTE1 and GSTL2 was also assessed. GST was the negative control in these experiments, and E1 was the positive control. The association between HPV16 L2 and E2 has not previously been shown, but as BPV1 L2 causes the relocation of E2 to ND10 domains when both proteins are expressed together in cells, the association between 16L2 and 16E2 was also tested out of curiosity (Day et al., 1998). Full length E2 protein (43K) only bound strongly to GSTL2 in this experiment, although weak binding of full length E2 to GSTE1 was also detected (Figure 3.12a).

The interaction between HPV16 E1 and E2 was originally identified using GSTE1, so it is unlikely that the N-terminally fused GST protein is preventing the E1-E2 interaction in this assay. However, in the pull-downs performed by Storey *et al*, no detergent was present in the pull-down buffer, and the binding of E2 to E1 was detected by autoradiogaphy, which is potentially a more sensitive method of protein detection than Western blotting (Storey et al., 1995). This may explain why the interaction between full length E1 and E2 was not observed in these experiments, which were performed in the

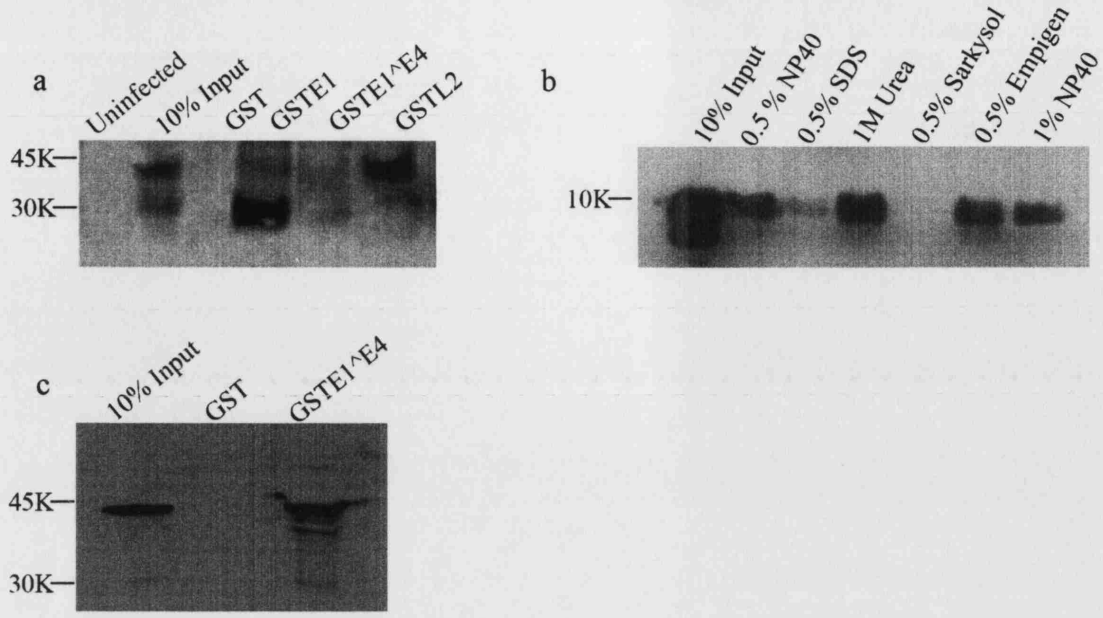


Figure 3.12 GST/ GST fusion protein pull-downs with E2
(a) Western blot with TVG261 of E2 eluted from GST and GST fusion protein pull-downs with RIPA extract of MCF-7 cells containing E2. (b) Western blot with TVG402 of His-E1^E4 eluted from GSTE2 pull-downs, performed in the presence of different detergents. (c) Western blot with TVG261 of E2 eluted from GST and GSTE1^E4 pull-downs with urea extract of MCF-7 cells containing E2.

presence of several detergents. Although curiously the E2 N-terminal form of E2 (E2N, 30K), did associate strongly with GSTE1.

A possible explanation for the failure to observe an interaction between E2 and E1^E4 in these latter experiments could also be due to differences in buffer composition. In this experiment E2 was extracted from cells using RIPA buffer, which contains 0.1 % (w/v) SDS and although this means the final concentration of SDS in the pull-down is ~0.003 %, it is difficult to reduce the local concentration of SDS around a protein by just dilution. To test the effect of different types of detergents/denaturants on the E2-E1^E4 interaction, GSTE2 was used in pull-downs with His-E1^E4, in the presence of SDS, and other detergents (Figure 3.11e). After comparison of the amount of E1^E4 that was seen following pull-downs in different detergents, it was found that SDS and sarkosyl appeared to disrupt the interaction, whereas the other agents tested had little effect (Figure 3.12b). Sarkosyl essentially behaves similarly towards proteins as SDS when used above its critical micelle concentration (0.42 %) and so it is not surprising like SDS, sarkosyl inhibits E2/E1^E4 association. In contrast, the interaction between E2 and E1^E4 was not found to be perturbed by the presence of a concentration of 1 M urea in the pull-down buffer, and so the GSTE1^E4 pull-down was repeated with urea extracted E2. This experiment confirmed that the association between E2 and E1^E4 was strongly hydrophobic, and was not due to a conformational change induced in E2 by the GST N-terminal fusion (Figure 3.12c). In support of the E2/E1^E4 interaction, E2 was co-immunoprecipated from E2/E1^E4 expressing cells using an E1^E4 antibody, but not from control cells by Dr. Qian Wang (data not shown).

3.12 HPV18 E2 associates with E1^E4

To assess whether the interaction between E2 and E1^E4 was unique to HPV16 or occurred in other HPV types, GST18E2 was used in a pull-down with His-18E1^E4 (Figure 3.13a). In this experiment both proteins were shown to interact directly, in addition the ability of 18E2 to associate with 16E1^E4 and 16E2 to associate with 18E1^E4 was also compared (Figure 3.13). In this pull-down 16E1^E4 was found to associate with 18E2, but only a small proportion of the input protein bound to

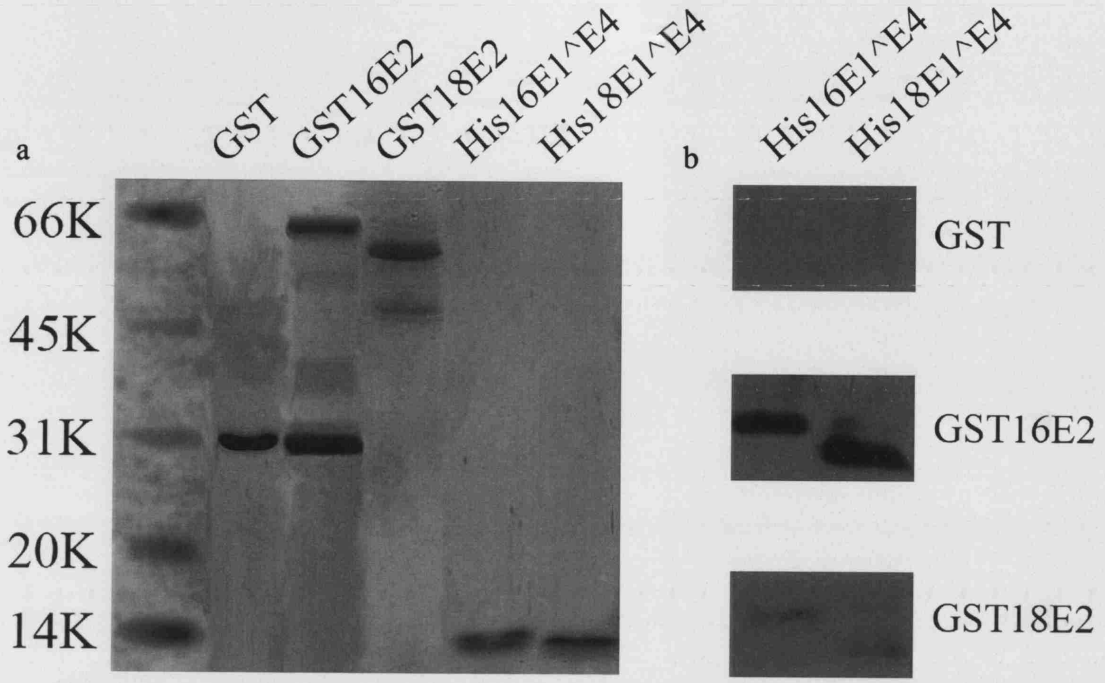


Figure 3.13 Interactions between 18E2 and E1^E4 proteins
(a) Purified proteins (50 % of input) as labelled separated by SDS-PAGE and then silver-stained respectively (The expression vector pGEX.18E2 was a gift from Dr. Françoise Thierry, Pasteur Institute, Paris). (b) Western blots with α-His tag of E1^E4 eluted from GST18E2 protein following pull-downs with His-16E1^E4 and His-18E1^E4 protein.

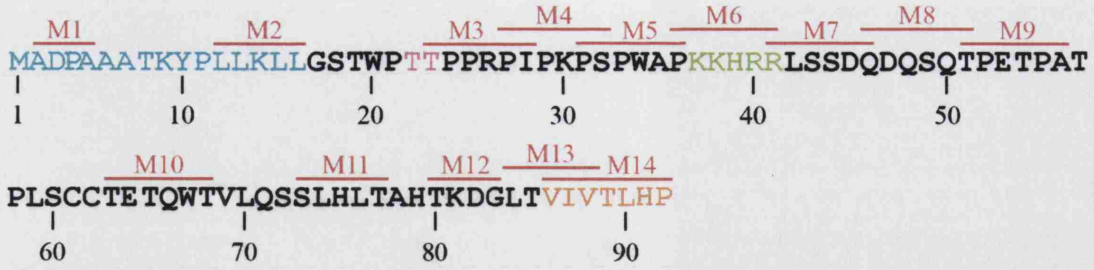
3.13 The production of 16E1^E4 deletion mutants

To determine the region of E1^E4 that is necessary for binding to E2, E1^E4 deletion mutants were produced in *E.coli*, and were tested for binding to E2, in the established pull-down assay (Section 2.6.2.6). E1^E4 sequences with sequential 4 to 7 amino acid deletions along the length of 16E1^E4, were available (Roberts et al., 1997). The positions of the amino acid deletions in the different mutant proteins (M1-M14) are illustrated in the context of full length E1^E4 in Figure 3.14. These mutant E1^E4 ORFs were amplified by PCR, the reverse primers that were used for the PCR were designed to recognise the 3'-end of the E1^E4 ORF but did not the terminal stop codon (Section 2.3.2.14). This allowed the mutated ORFs to be sub-cloned into the bacterial expression vector (pET-28a+), in-frame with the His-tag, so that C-terminally His-tagged E1^E4 mutant proteins could be produced (this was achieved with the help of Dr.Deb Jackson, NIMR).

Bacterial cultures of M1-M14 and WT E1^E4 were grown O/N, before being diluted 1 in 100 in LB-AMP. The cultures were then grown for another 2 h, before being induced with IPTG, for another 2 h. A fraction of these cultures (20 μl) was taken and Western blotted with the polyclonal antibody, α-MBPE1^E4. All the constructs with the exception of pET.M2 appeared to express E1^E4 proteins, following induction but at varying levels (Figure 3.15). To test whether the M2 protein was expressed, but was just not recognised by the rabbit polyclonal antibody used, the sample was Western blotted with another E1^E4 antibody (TVG402), and with an antibody which recognised the C-terminal His-tag that was fused to all the E1^E4 proteins. M2 was detected using both of these antibodies (Figure 3.15).

The mutant E1^E4 cultures were prepared simultaneously to minimise variation between preparations, and using conditions that had been optimised for the production of WT E1^E4.

a



Key for coloured Amino acids

- Amino acids required for keratin targeting
- Amino acids essential for cyclin B binding
- Putative Nuclear Localisation Sequence (NLS)
- Amino acids required for oligomerisation

b

MUTANT NAME	POSITION OF Δ AMINO ACIDS	Δ AMINO ACIDS
M1	2-6	ADPA
M2	12-16	LLKLL
M3	23-28	TPPRPI
M4	27-32	PIPKPS
M5	31-36	PSPWAP
M6	36-41	PKKHRR
M7	41-46	RLSSDQ
M8	46-51	QDQSQT
M9	51-56	TPETPA
M10	63-68	TETQWT
M11	73-77	SLHLT
M12	80-83	TKDG
M13	84-88	LTVIV
M14	86-92	VIVTLHP

Figure 3.14 HPV16 E1^E4 deletion mutants
(a) Primary sequence of HPV16 E1^E4. Amino acids that have been shown to be important for binding to cellular proteins and self-association are highlighted (Davy et al., 2002; Roberts et al., 1994; Roberts et al., 1997; Wang et al., 2004). Above the sequence, is shown the positions of the E1^E4 deletion mutants (M1-M14) (b) Table of the position and residues deleted in the various His-E1^E4 mutant proteins.

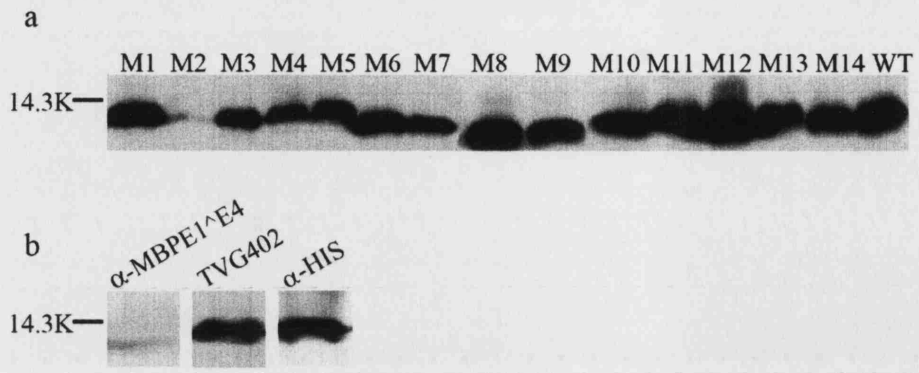


Figure 3.15 Expression of His-E1^E4 mutants
(a) Western blots of IPTG-induced bacterial cell extracts, containing mutant E1^E4 proteins. The first panel was probed with α-MBPE1^E4. (b) Western blot of M2 extract probed with E1^E4 specific antibodies.

This method resulted in the isolation of E1^E4 proteins but the preparations seemed to contain bacterial contaminants. To overcome this problem, the proteins that bound to metal-affinity beads were washed with urea buffer at a lower pH (pH 6.0) than the binding buffer (pH 7.0) briefly before elution. This strategy significantly reduced contamination, but it also decreased the yield of E1^E4 proteins significantly. In an attempt to reduce contaminants but without compromising yield the proteins were prepared but with additional neutral pH urea buffer washing steps, and were eluted in three sequential fractions at pH 4.0. Contaminated E1^E4 was eluted off first, but the second and third fractions also contained E1^E4, but with little or no contamination (Figure 3.16a).

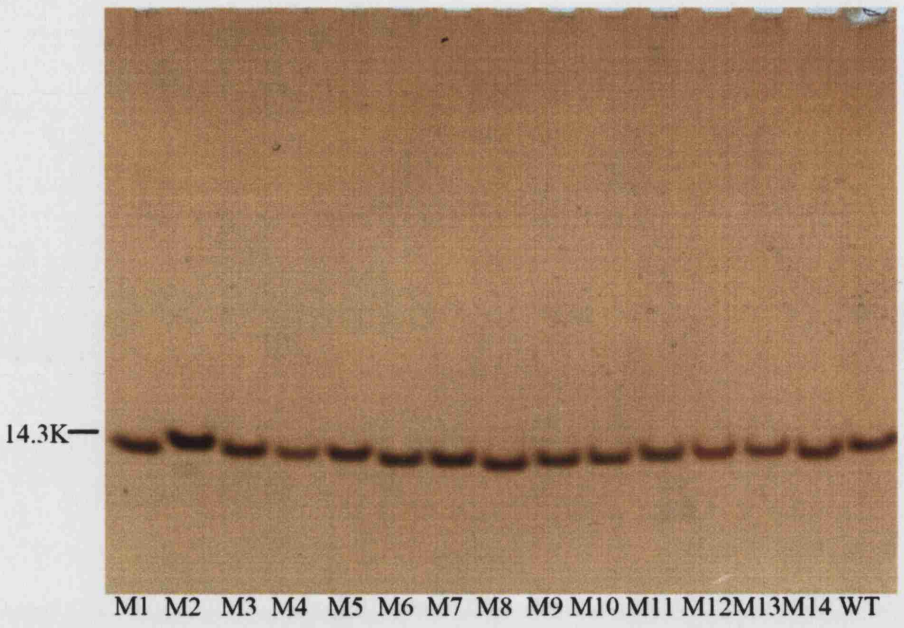
The different E1^E4 mutants that were produced, had only small differences in molecular weight (MW) but migrated differently when separated on a 4-20 % polyacrylamide gel. The differences are more pronounced in Figure 3.16b when the proteins were separated further on a 15 % polyacrylamide gel. M2 migrates at a higher MW than the other proteins on the gel, and M6, M7, M8 and M9 migrate slightly below the other mutants and WT proteins. It is thought that these effects on the migration of E1^E4 mutants may be reflect structural defects induced in the whole protein as a result of the deletion of key structure retaining residues.

3.14 The binding efficiencies of different E1^E4 mutants for E2

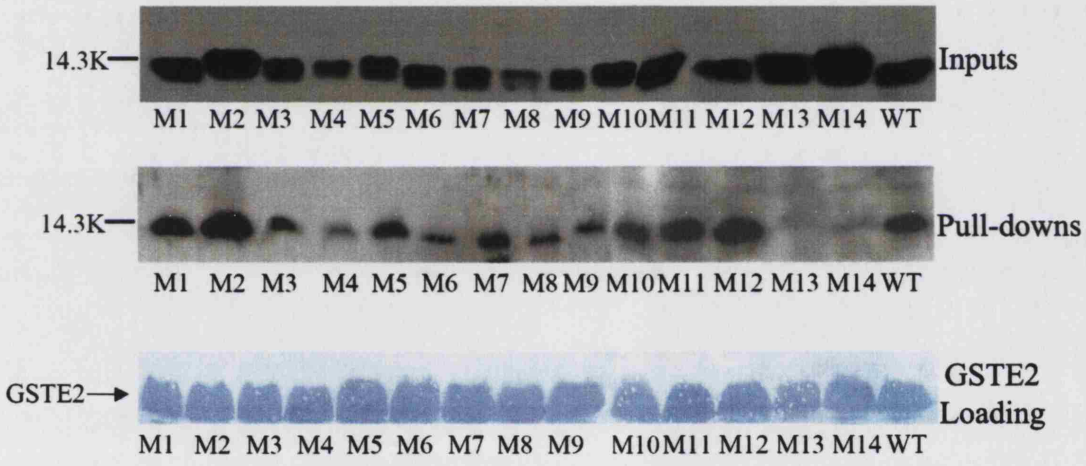
To enable equal quantities of the purified His-tagged proteins to be used in parallel pull-downs between E2 and the different mutants and WT proteins, the OD of the preparations at a wavelength of 280 nm were measured. On average the yield of protein was 43 ± 3 μg ml^{-1}, for M3-M14 and WT E1^E4, but was significantly higher for M1 and M2, which yielded on average 235 ± 15 μg ml^{-1} of protein, based on the OD_{280}. To enable approximately equal quantities of all E1^E4 proteins to be used in pull-downs, some of the preparation had to be diluted prior to use. The samples were then Western blotted to check for equal protein loading, and when appropriate the sample concentrations were readjusted accordingly. Despite this, it was a formidable task to adjust the protein levels in WT and all 14 mutants so that the amount used

Figure 3.16 Identification of E1^E4 residues that are critical for E2 binding
(a) Silver-stained gel of 2nd or 3rd elutions of M1-M14 and WT His-E1^E4 proteins from a metal-affinity column. (b) The first two panels are anti-His Western blots of input and eluted E1^E4 proteins of a representative GSTE2 pull-down experiment. The last panel is a Coomassie blue-stained Immobilon membrane showing the amounts of GSTE2, used in the corresponding pull-down experiment. (e) Graph comparing the relative binding of E2 to E1^E4 mutants and WT E1^E4. The densitometry values of the proteins eluted were standardised with respect to the densitometry values of the protein inputs, for each experiment. All the pull-downs were repeated three times, and the graph reflects the average binding between each mutant protein and GSTE2. The error bars show the SE.

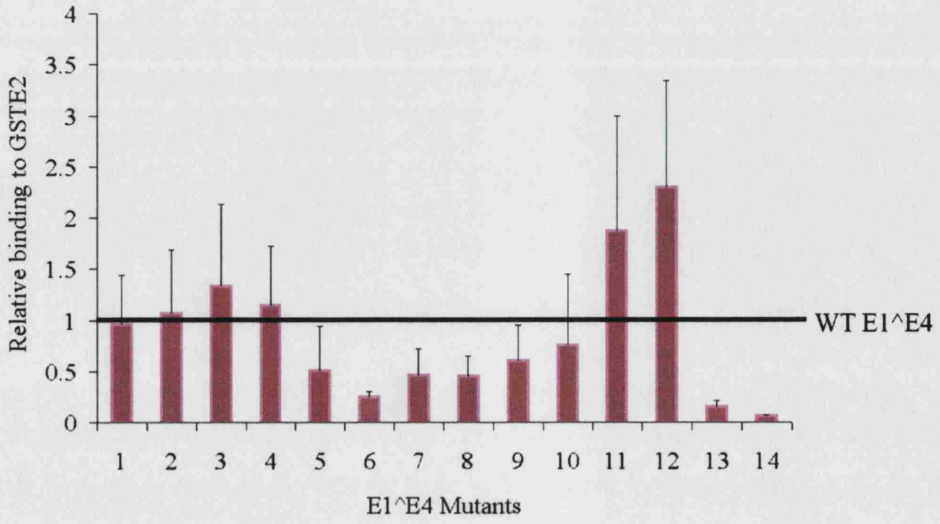
a


14.3K
M1 M2 M3 M4 M5 M6 M7 M8 M9 M10 M11 M12 M13 M14 WT

b


14.3K
Inputs
M1 M2 M3 M4 M5 M6 M7 M8 M9 M10 M11 M12 M13 M14 WT
14.3K
Pull-downs
M1 M2 M3 M4 M5 M6 M7 M8 M9 M10 M11 M12 M13 M14 WT
GSTE2
GSTE2 Loading
M1 M2 M3 M4 M5 M6 M7 M8 M9 M10 M11 M12 M13 M14 WT

c


Relative binding to GSTE2
4
3.5
3
2.5
2
1.5
1
0.5
0
WT E1^E4
1 2 3 4 5 6 7 8 9 10 11 12 13 14
E1^E4 Mutants

in each pull-down was exactly equal. Protein concentrations can be estimated by measuring the absorbance of tyrosines and tryptophan residues in a protein solution at a wave length of 280 nm. However, the presence of DNA and higher order protein structures in a solution may affect molar absorption, the latter is sensitive to small differences in pH and ionic strength, which may explain the difficulties experienced in trying to quantify different E1^E4 protein preparations by spectral absorbance alone.

Despite small differences in protein loading pull-downs were performed three times with each mutant protein. The results of a representative pull-down experiment between GSTE2 and the WT and mutant E1^E4 proteins are shown after Western blotting with an α-His-tag antibody which recognises all the E1^E4 proteins used. Input GSTE2 and His-tagged proteins used for each pull-down are also shown (Figure 3.16b). In each pull-down experiment, the relative binding of each mutant E1^E4 (and also WT E1^E4) to E2 was assessed with respect to the amount of input by measuring band density. Comparisons of the relative binding efficiencies of the different mutants to GSTE2 are shown relative to WT in Figure 3.16c. The central portion and the extreme C-terminus of E1^E4 both showed reduced binding to E2, in particular M6 (Δ36-41), M13 (Δ84-88) and M14 (Δ86-92). This suggests that potentially two regions of E1^E4 may be involved in mediating the E2-E1^E4 interaction. However, the interpretation of this result is made more complicated when the residues implicated are considered in the context of the E1^E4 protein.

M6 is a six amino acid deletion which incorporates four positively charged residues and one proline residue (Figure 3.14b). The altered migration of M6 protein following SDS-PAGE, suggests that the amino acids deleted from E1^E4 to produce the mutant M6 E1^E4 protein may affect its structural integrity and thus in turn possibly its ability to associate with E2 (Figure 3.16a and b). Indeed M7 and M8 which superficially appear to have deletions that affect E1^E4 structure, also seem to bind E2 with reduced capacity when compared to WT E1^E4. Previous experiments which have characterised the nature of the E2-E1^E4 interaction are also in disagreement with the finding that the charged sequence (36-41) may be critically involved in E2 binding, as they suggest the interaction between E2 and E1^E4 is strongly hydrophobic and thus unlikely to involve charged residues (Figure 3.11f and 3.12b). M13 and M14 cover 9 residues of E1^E4 together, but overlap by 3

hydrophobic residues (VIV) suggesting these three amino acids may form part of the site that is involved in mediating the E2-E1^E4 interaction. However, these residues have also been shown to be critical for E1^E4 multimerisation, so alternatively this binding data could mean that E1^E4 needs to be in a multimeric state to associate with E2.

3.15 Discussion

To allude to some of the functions of E1^E4 in the virus life cycle, its binding ability to cellular and HPV proteins was tested. Preliminary experiments suggested that E1^E4 could associate with several cellular proteins which are expressed in HPV-transformed cervical epithelial cells. The actual proteins that formed a complex with E1^E4 were not identified in this study, although based on their sizes, the majority of these proteins are likely to be novel interactions. In addition, E1^E4 was shown to associate directly for the first time with the early proteins E7 and E2, and the viral capsid proteins, L1 and L2. These HPV proteins are thought to be expressed in different layers of the epithelium, where they contribute to different stages of the HPV life cycle.

E1^E4 expression coincides with the onset of HPV replication and extends into the upper layers where virions are packaged and released (Breitburd et al., 1987; Doorbar et al., 1997). Roles for E1^E4 have been suggested in both genome amplification and release, which are independent of E1^E4 association with HPV proteins (Bryan and Brown, 2000; Davy et al., 2002; Raj et al., 2004; Wang et al., 2004). However, the interactions identified here may contribute, as HPV proteins tend to target more than one route to bring about an effect. For example E7 promotes S-phase by reducing RB transcription and also by targeting the RB protein for degradation (Dyson et al., 1989; Zerfass et al., 1995). E1^E4 is thought to functionally cooperate with E2 in viral replication and virion egress, and so it is quite possible that the direct association of E1^E4 with the transcription/replication factor E2, and the structural capsid proteins L1 and L2 may directly contribute to these functions (Yang et al., 1991; Desaintes et al., 1999; Raj et al., 2004). E1^E4 is expressed together with E7 and E2 at the onset of genome amplification and also in the upper layers of the epithelium where its expression overlaps with L1 and L2. Thus, these novel interactions are likely to occur

in different layers of the epithelium. There was a distinct preference displayed for the unphosphorylated form of E1^E4 by E1^E4 itself, E7 and L2, whereas E2 and L1, appeared to bind to both forms of E1^E4 strongly. This subtle difference in binding ability between the different HPV proteins to E1^E4 may enable E1^E4 to cooperate with different proteins simultaneously.

Another interesting finding was that 16L2 could associate with 16E2, as well as E1^E4. BPV1 L2 has previously been shown to cause the relocation of E2 to ND10 domains (Day et al., 1998). Although the mechanism by which this occurs has not been elucidated, it has been speculated that this may involve a direct association between the two proteins (Day et al., 1998). The identification of a direct association between E2-L2 in this thesis, and by others adds weight to this hypothesis (Heino et al., 2000; Okoye et al., 2005). The association between E2 and L2 is thought to facilitate packaging of the replicated viral genomes into particles, and it is also possible that the ability of E1^E4 to associate with E2, L2 and L1 may enhance this process (Zhao et al., 2000). Hypothetically E1^E4 may even participate in a complex with E2, L2 and L1, which together may increase the stability of the virion.

This investigation has led to the identification four novel E1^E4 interactions which could be significant in the context of the HPV life cycle or even in the prevention of malignant progression, as the E4 ORF has commonly been observed to be lost in cancers. However, it was decided to focus on the E2-E1^E4 interaction for the rest of this investigation. HPV18 E2 and E1^E4 proteins were also shown to interact, and so were but not as strongly as the interaction between 16E2 and E1^E4 proteins. This shows that the association between E2 and E1^E4 is not exclusive to HPV16. The fact that 18E2 and 16E2 were capable of associating with both 16E1^E4 and 18E1^E4 also suggests the binding sites on the two proteins are conserved to an extent between HPV types.

The preliminary pull-down experiments suggested that the extreme N-terminus of the E1^E4 protein was not involved in mediating its binding to E2, which was confirmed in pull-down experiments using E1^E4 deletion mutants. Instead, amino acids in the central (36-41) and the C-terminal (86-88) region of E1^E4 were implicated in E2 binding, based on the reduced ability of E1^E4 proteins with these regions deleted to

associate with E2. However, just because the deletion of both these regions of E1^E4 disrupts E2 binding *in vitro*, this does not necessarily mean they are involved in binding, as the deletion of residues from a protein can gravely affect protein structure and function. For this reason further investigation is required to identify the E1^E4 residues that are critical for mediating the E1^E4-E2 association. However, as the interaction between the two proteins appears to be strongly hydrophobic, of the two regions identified as possible binding sites, the hydrophobic C-terminus seems to be the favoured candidate.

4 Chapter 4 E1^E4 causes the cytoplasmic retention of E2, and can affect HPV early gene transcription

4.1 Introduction

An *in vitro* interaction between E2 and E1^E4 was described in the previous chapter. However, as 16E1^E4 is predominantly a cytoplasmic protein, and 16E2 is predominantly nuclear, it is not obvious where in the cell this interaction would occur, or indeed whether it would occur at all (Doorbar et al., 1992; Sanders et al., 1995).

The co-expression of HPV proteins that interact *in vitro* have been shown to cause a change in the localisation of one of the two proteins in cultured cells. For example, L2 can cause the relocation of L1 to ND10 domains, and similarly E1, can cause E2 accumulation at these sites (Day et al., 1998; Swindle et al., 1999; Florin et al., 2002). In these experiments, the colocalisation of the proteins was not always apparent in all the transfected cells, but was detected in subpopulations of cells. This suggests that other factors, apart from the ability of the proteins to interact, may influence the ability of the proteins to colocalise in cells. In the case of E1 and E2, the phase of the cell cycle seemed to be important for colocalisation. The redistribution of HPV proteins in cells following co-expression may be important for the progression of the virus life cycle. For example, the relocation of L1 and E2 to ND10 domains by L2 is thought to facilitate genome packaging. The translocation of proteins that are thought to be involved in packaging to ND10 domains, in effect increases the local concentration of components involved in genome packaging to specific sites in the cell, and in this way facilitates the process of encapsidation. To investigate the localisation of E2 and E1^E4 in cell culture following co-expression of the two proteins it was decided to use immunofluorescence, analogous to E2 and L2 as well as E1 and E2 co-expression studies (Day et al., 1998; Swindle et al., 1999).

It can be interpreted from a study by Demeret *et al*, concerned with the transactivational affect of E1 on E2-mediated transcription that E1^E4 may play a role in the transcriptional regulation of the early promoter (Demeret et al., 1998). In this study E1 and E2 were co-expressed from a cassette of the early region of the HPV genome, which contained E1 and E2 ORFs as well as E7, E1^E4 and E5. The

authors found that this cassette expression system caused higher levels of transactivation than assays where E1 and E2 were expressed from separate expression constructs. This could be due to more efficient E1 synthesis when expressed from the cassette system, as compared to expression from an E1 expression plasmid as proposed by the authors. However, it is possible that in addition to this the co-expression of E1, E2 and E1^E4 from the early cassette region may contribute to enhanced transactivation by the cassette compared to individual expression vectors, given that E2 and E1^E4 protein interaction has been shown. To address this possibility it was decided to use a similar transcription assay system as Demeret *et al*, to test the effect of E1^E4 on the transcription of a luciferase reporter gene regulated by the wild-type (WT) HPV16 long control region (LCR).

4.2 Expression patterns of E1^E4 and E2 in epithelial cells

Before E1^E4 and E2 phenotypes could be studied together, it was important to be able to recognise the expression patterns of the two proteins when expressed alone. In SiHa cells, E1^E4 showed three patterns of expression, all of which have been reported previously (Figure 4.1a). In the majority of cells E1^E4 had a filamentous pattern due to its colocalisation with the keratin filament network (Doorbar et al., 1991). In other cells E1^E4 formed perinuclear bundles, or displayed a punctate pattern in the cytoplasm, due to the association of E1^E4 with the collapsed keratin network or with mitochondria, respectively (Doorbar et al., 1991; Raj et al., 2004). The number of cells that show colocalisation between E1^E4 and mitochondria increases with time post infection, following the collapse of the keratin network. E2 showed a diffuse nuclear distribution in the majority of cells in which it was expressed, but a sub-population of E2 positive cells (16-20 %) displayed diffuse nuclear and cytoplasmic E2 expression patterns at both early (24 h) and late (72 h) time points post infection (Figure 4.1b).

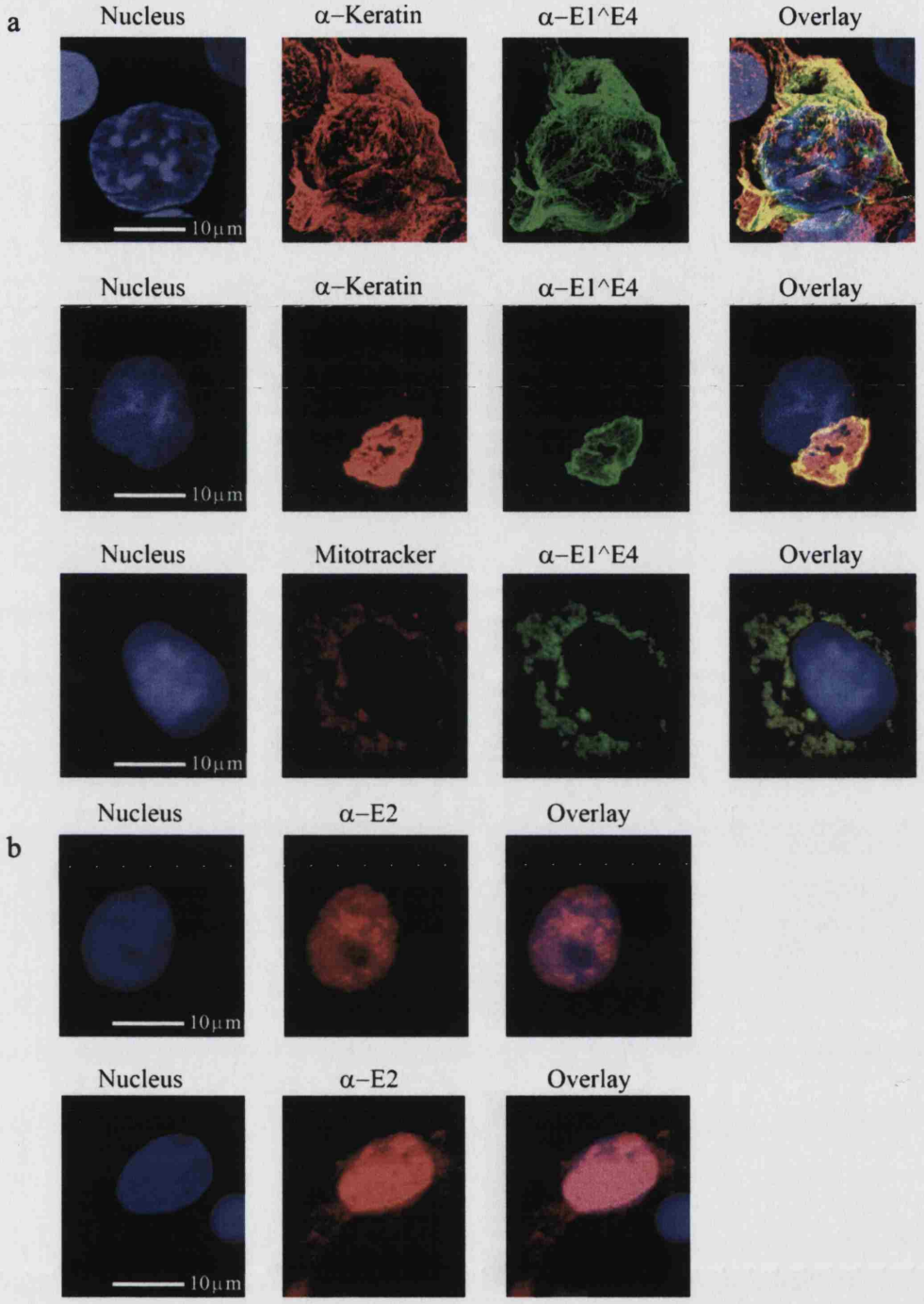


Figure 4.1 HPV16 E1^E4 and E2 immunofluorescent staining

SiHa cells were infected with rAd.E1^E4 or rAd.E2 for 24 h. E1^E4 was detected using the monoclonal E1^E4 antibody (TVG405) conjugated to a fluorescent fluorophore, and E2 by the monoclonal antibody TVG261 and an anti-mouse secondary antibody which was conjugated to a fluorescent fluorophore. The nuclei of the cells were counterstained with DAPI (blue). (a) Three E1^E4 (green) expression patterns are shown. In the first row E1^E4 is associated with the cytoplasmic keratin filament network (red), in the second row E1^E4 is associated with a collapsed filament network (red) and in the third row E1^E4 is associated with mitochondria (red). Keratins were detected using a pan anti-keratin antibody (Sigma) and mitochondria was detected by metabolic labelling of cells with Mitotracker® (Molecular probes). The E1^E4/keratin images were produced by Peter Laskey. (b) Two E2 expression patterns are shown. In the first row E2 is completely nuclear and in the second row E2 is expressed in both the nucleus and cytoplasm of the cell.

4.3 E1^E4 and E2 antibodies are not cross-reactive

To ensure that the antibodies that were used to detect E1^E4 and E2 proteins did not cross-react, SiHa cells were infected with recombinant adenoviruses (rAd) that expressed either E2 or E1^E4 and were then stained with monoclonal antibodies that recognised E2 (TVG261; Figure 4.2a) or E1^E4 (TVG405; Figure 4.2b). To allow two monoclonal antibodies to be used for double staining TVG405 was conjugated to an Alexa Fluor® 488 fluorophore (Molecular probes). This experiment showed that the antibodies that were used to detect E2 and E1^E4 were specific, as no detectable cross-reactivity was seen between the primary and secondary antibodies that were used to detect E2 and the E1^E4 protein or between the fluorophore conjugated monoclonal antibody that was used to detect E1^E4 and the E2 protein.

4.4 E2 accumulates at E1^E4 associated cytoplasmic structures

SiHa cells were co-infected with rAds expressing E1^E4 and E2 for a time course of 24-72 h. Recombinant protein expression was then detected by immunofluorescence and the cells were analysed by fluorescent microscopy. It was found that at early time points post infection that the majority of cells that co-expressed E2 and E1^E4 did so in different compartments of the cell, i.e. E2 was in the nucleus and E1^E4 was in the cytoplasm (Figure 4.4a). However, a sub-population of cells (28 %) expressed E2 and E1^E4 patterns in the cytoplasm (Figure 4.3a). In these cells E2 was abundant in the nucleus as well as the cytoplasm. Interestingly, in these cells cytoplasmic E2 conformed to the characteristic E1^E4 distribution within the same cell (Figure 4.4).

At later time points, the majority of cells expressing E1^E4 show E1^E4/mitochondria colocalisation this is presumably, following the collapse of the keratin network (Figure 4.1a). Importantly, the cytoplasmic colocalisation of the E1^E4 and E2 proteins to mitochondria was observed at a higher frequency at late time points i.e. 46-48 % of the population (Figure 4.3a 4.4b and c). The majority of cells co-expressing E2 and E1^E4 still contained nuclear E2, although, some cells (~5 %) at 72 h post infection had only cytoplasmic E2 which colocalised perfectly with E1^E4 (Figure 4.4c). This suggests that E1^E4 is causing the relocation of E2 out of the nucleus. In support to this hypothesis, the proportion of cells (14-18 %) showing cytoplasmic

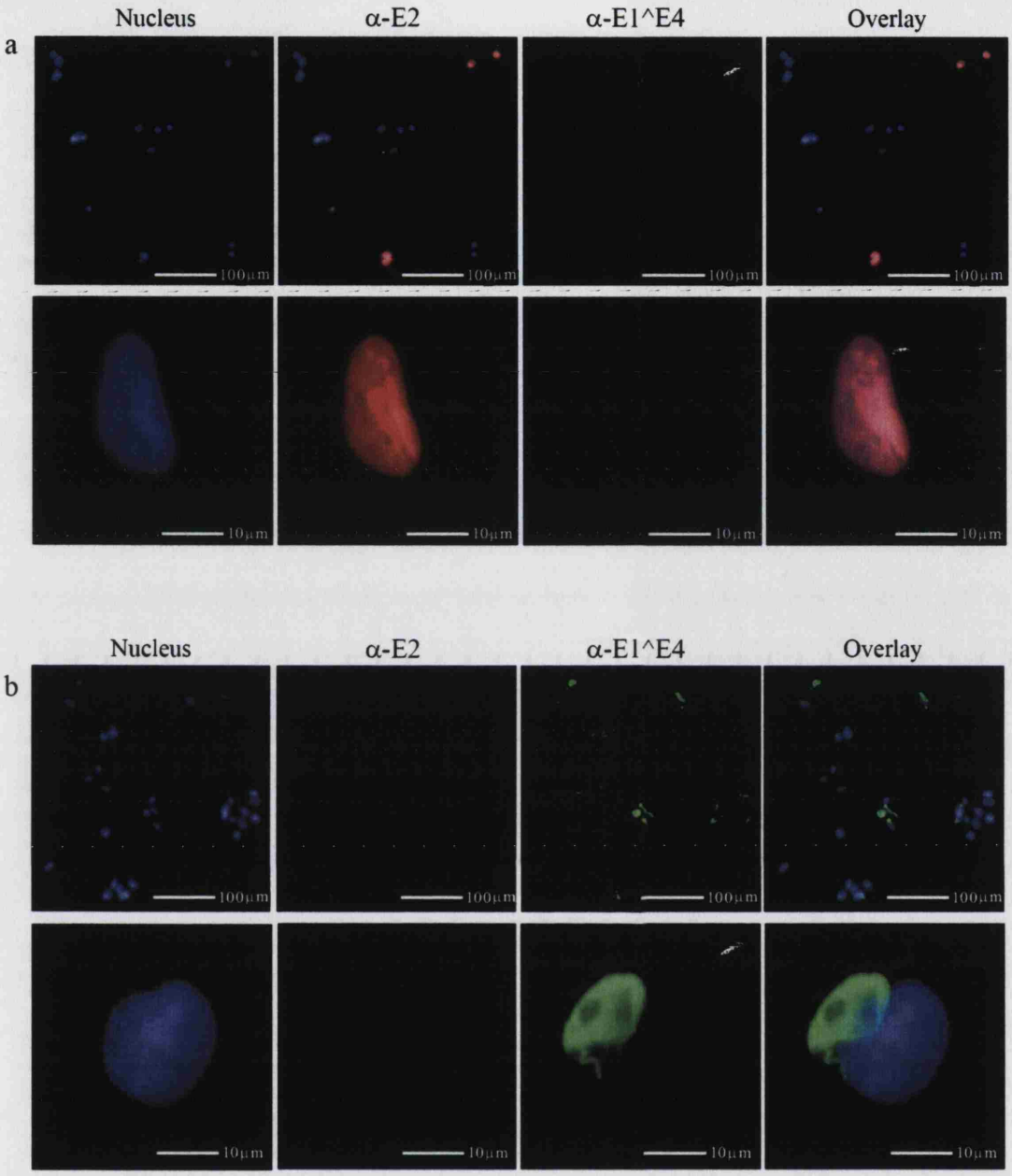


Figure 4.2 Testing TVG405 and TVG261 cross-reactivity
SiHa cells were infected with rAd.E1^E4 or rAd.E2 for 24 h. Cells were double-stained with the E1^E4-specific antibody, TVG405 and the E2-specific antibody, TVG261. The nuclei of the cells were counterstained with DAPI (blue). Nuclear staining and the staining patterns of TVG261 and TVG405 are shown separately and altogether (overlay) at the same exposure, at 10x or 100x magnification. (a) SiHa cells expressing E1^E4. (b) SiHa cells expressing E2.

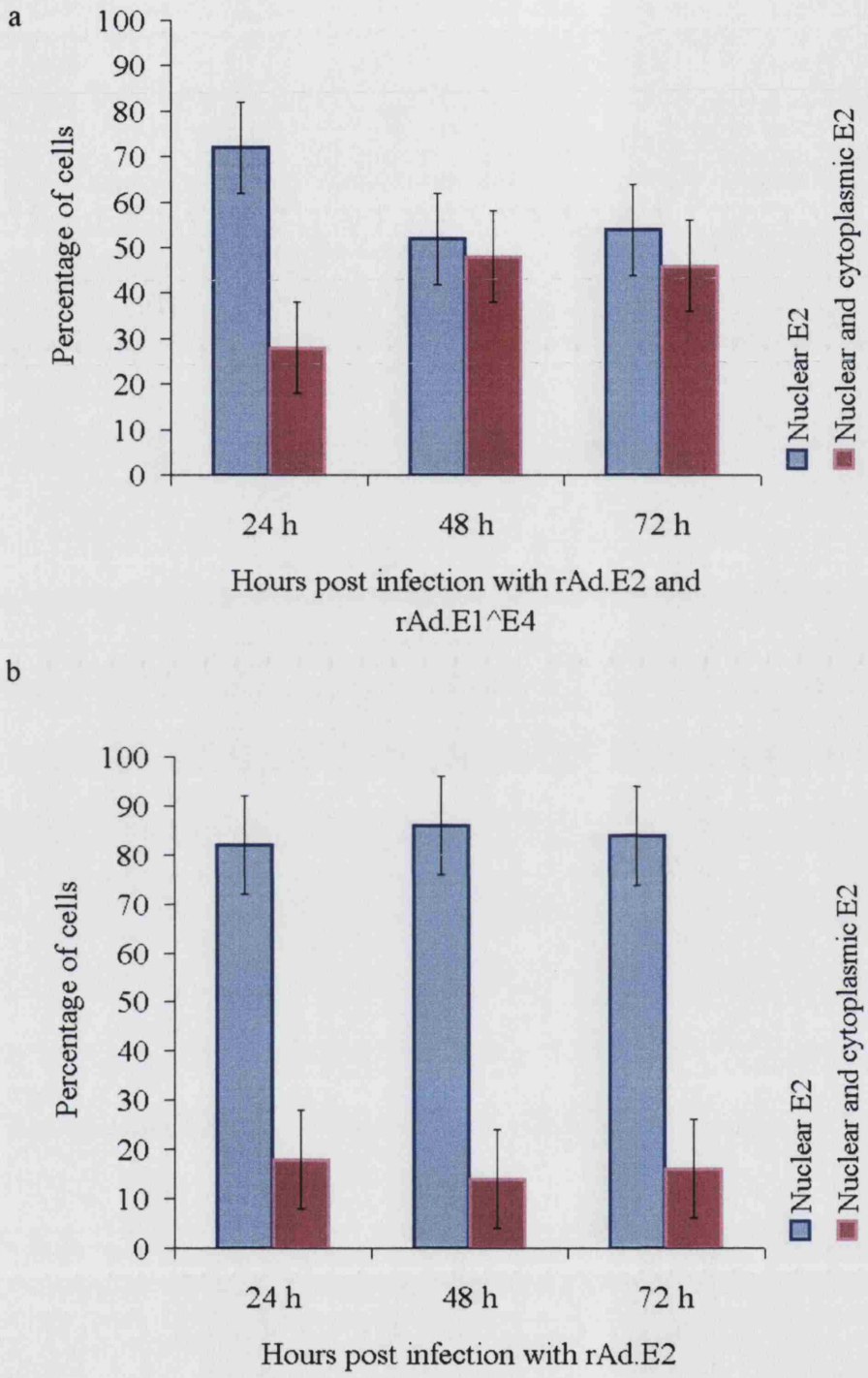


Figure 4.3 Comparison of the cytoplasmic accumulation of E2 in the absence and presence of E1^E4

SiHa cells were infected with rAds expressing E2 and β-gal or E2 and E1^E4. The cells were then stained by immunofluorescence for E2, using the antibody TVG261 and also E1^E4, with the antibody TVG405 where appropriate. Bar charts represent the frequency of cytoplasmic E2 at three time points post-infection. On average 40 infected cells were counted for each time point. Error bars of ±10 % are shown on each graph. (a) SiHa cells infected with rAd.E2 and rAd.E1^E4. (b) SiHa cells infected with rAd.E2 and rAd.βGal.

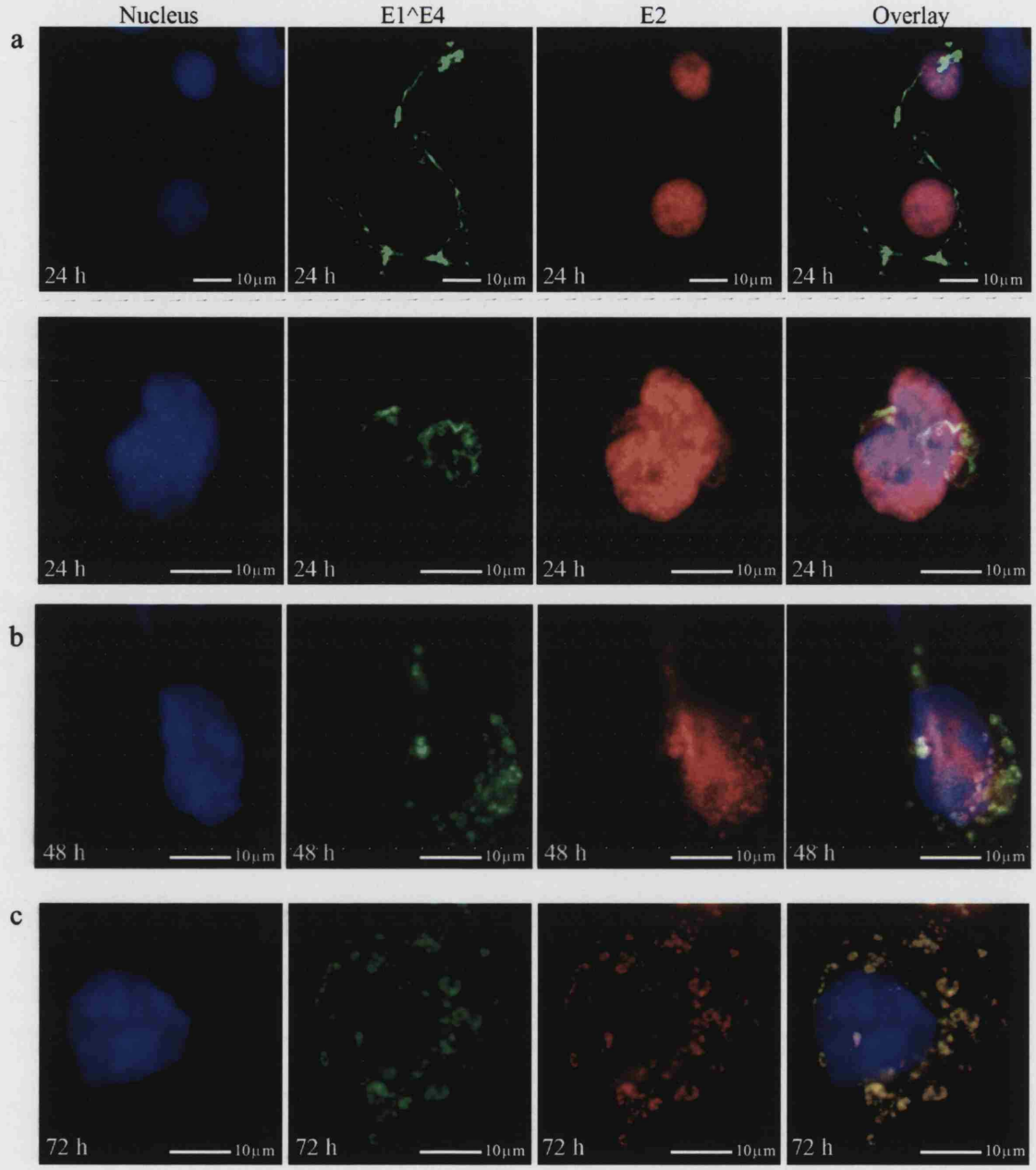


Figure 4.4 Co-expression of E2 and E1^E4 in SiHa cells using rAds
SiHa cells were infected with rAd.E1^E4 and rAd.E2. The E1^E4 and E2 proteins were detected by immunofluorescence using the antibodies TVG405 and TVG261, respectively. The nuclei of the cells were counterstained with DAPI. Nuclear (blue), E1^E4 (green) and E2 (red) staining of cells is shown separately and altogether (overlay) at the same exposures. (a) Cells infected for 24 h. (b) Cells infected for 48 h. (c) Cells infected for 72 h.

E2, when E2 was expressed in the absence of E1^E4 does not change significantly with time (Figure 4.3b).

Despite high levels of E2/E1^E4 colocalisation being observed in cells co-expressing both proteins at late time points post-infection, the remaining half of the population displayed no significant levels of E2/E1^E4 colocalisation. This suggests other factors in addition to the ability of the proteins to interact, may influence the accumulation of E2 to E1^E4-structures in the cytoplasm *in vivo*. In fact, even though E2 has been shown to be a predominantly nuclear, it has been detected in the cytoplasm in the upper layers of HPV16 infected lesions (Maitland et al., 1998). This observation suggests that differentiation could increase the propensity of the E2-E1^E4 interaction. To investigate this possibility it was decided to look at the expression of E2 and E1^E4 in raft culture and in low-grade lesions.

4.5 Immunostaining of raft sections

Raft and tissue sections are formalin fixed and paraffin embedded to preserve the tissue morphology. This process can alter the structure of proteins and result in the masking or loss of immunological reactive epitopes on the protein surface. The E1^E4 antibody, TVG405 has been used to stain tissue sections previously, suggesting the epitope recognised by this antibody is resistant to the fixation process (Doorbar et al., 1997; Middleton, 2003). For this reason TVG405 was used to detect E1^E4 specifically in HPV positive raft sections (which were kindly provided by Tomomi Nakahara, University of Wisconsin, Madison, Wisconsin). In raft sections E1^E4 was detected in some cells in the upper layers of the epithelium (Figure 4.5).

In contrast, the detection of E2 in raft sections was not as straight forward. The same antibody that was used to detect E2 in cell culture was tested on raft sections. This resulted in very weak nuclear staining of almost all the cells of the HPV positive and negative section, suggesting the fluorescent signal detected was non-specific (Figure 4.6a). Maitland *et al* has convincingly shown specific E2 staining of low-grade HPV16 lesions using a rabbit polyclonal antibody raised against the C-terminus of

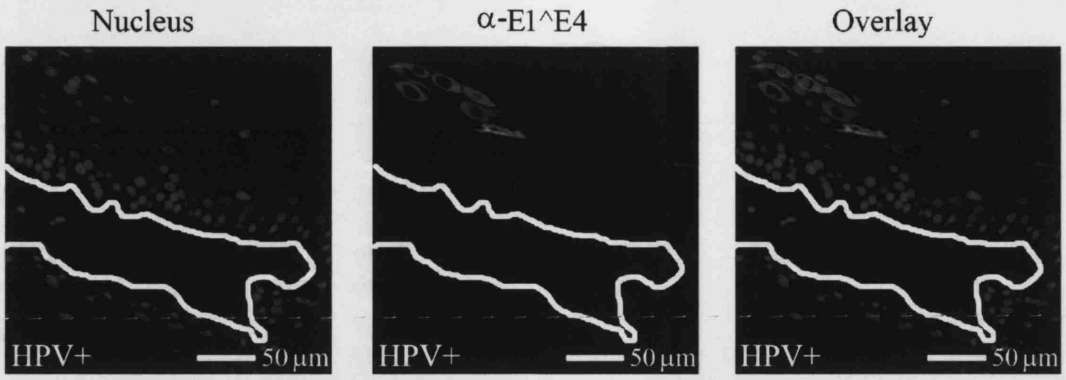


Figure 4.5 E1^E4 staining of raft sections
HPV16/pNeo transfected (HPV+) and rafted NIK cells were immunofluorescently stained with Alexa-488 conjugated TVG405. The position of the basal layer is indicated on each panel by a white line.

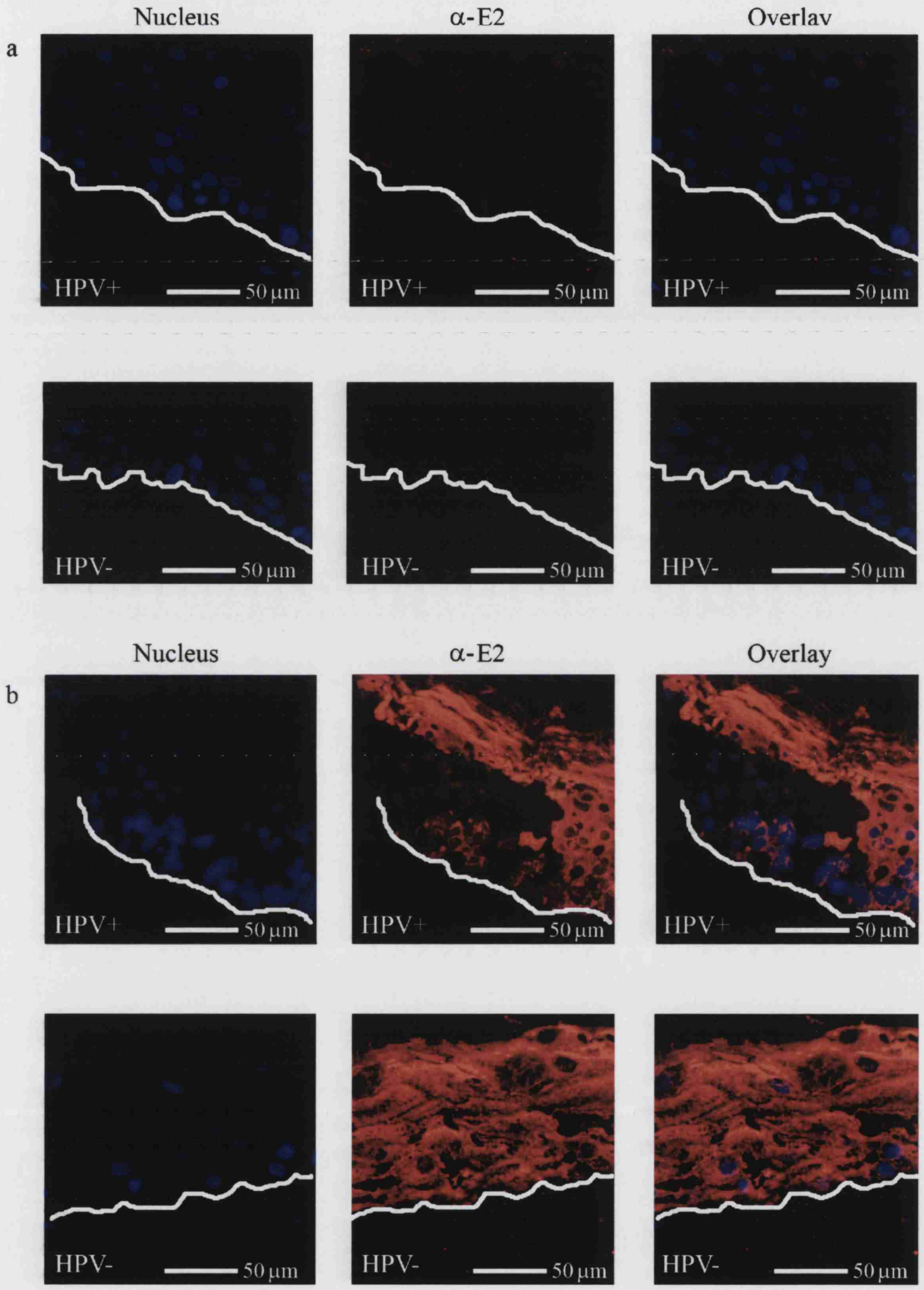


Figure 4.6 E2 staining of raft sections
HPV16/pNeo transfected (HPV+) or only pNeo transfected (HPV-) and rafted NIK cells were immunofluorescently stained with E2 antibodies. The position of the basal layer is indicated on each panel by a white line. (a) Purified TVG261 was used for staining. (b) Rabbit polyclonal antibody raised against the E2 C-terminus was used for staining.

16E2 (E2C) (Maitland et al., 1998; Stevenson et al., 2000). For this reason this antibody was requested from Maitland *et al.* The antibody received had been used in the previous published studies but following affinity purification, unfortunately we were only provided with a small volume (100 µl) of E2C rabbit serum that had not been affinity purified. We were unsure of the concentration of E2C-specific antibody this contained and so we did not attempt to affinity purify it ourselves. The rabbit serum was incubated with acetone powders of human skin to try and reduce the possibility of non-specific staining of raft sections. Despite this, in our hands this antibody appeared to stain the cytoplasm of cells non-specifically even when it was used at half the recommended dilution (Figure 4.6b) (Maitland et al., 1998).

Another rabbit polyclonal antibody which also recognised the C-terminus of 16E2 (a kind gift from Dr. Kevin Gaston, University of Bristol, Bristol, UK) was also tested under similar conditions. This antibody was also found to stain HPV positive and negative raft sections, suggesting the staining patterns obtained were not E2-specific (data not shown). It was not clear from the previous experiments why the staining of HPV positive raft sections did not work. Was it because the antibody/staining methods used were not optimal or alternatively was it because E2 was poorly expressed in the raft culture? To test the latter possibility, E2 and E1^E4 staining of a CIN I lesion was performed, as it has been documented separately that HPV16 low grade lesions express E1^E4 and E2 in the upper layers at high levels (Doorbar et al., 1997; Stevenson et al., 2000). As expected E1^E4 was not uniformly present throughout the lesion, but was only detected in the superficial and intermediate layers of HPV positive parts of the epithelium (Figure 4.7a).

The E2 staining patterns of the presumed HPV positive and negative regions of the lesion were similar at low magnification. However, when examined at a higher magnification, the intensity of the E2 stain was higher than the apparent background fluorescence in some of the cells in the upper layers of the HPV positive section but not in the HPV negative section (Figure 4.7b). This could correspond to cells displaying what appears to be specific E2 staining, an example of a cell that shows nuclear E2 and another that appears to have both nuclear and cytoplasmic E2 are arrowed in Figure 4.7b. E1^E4 is also expressed in the latter cell, in which it

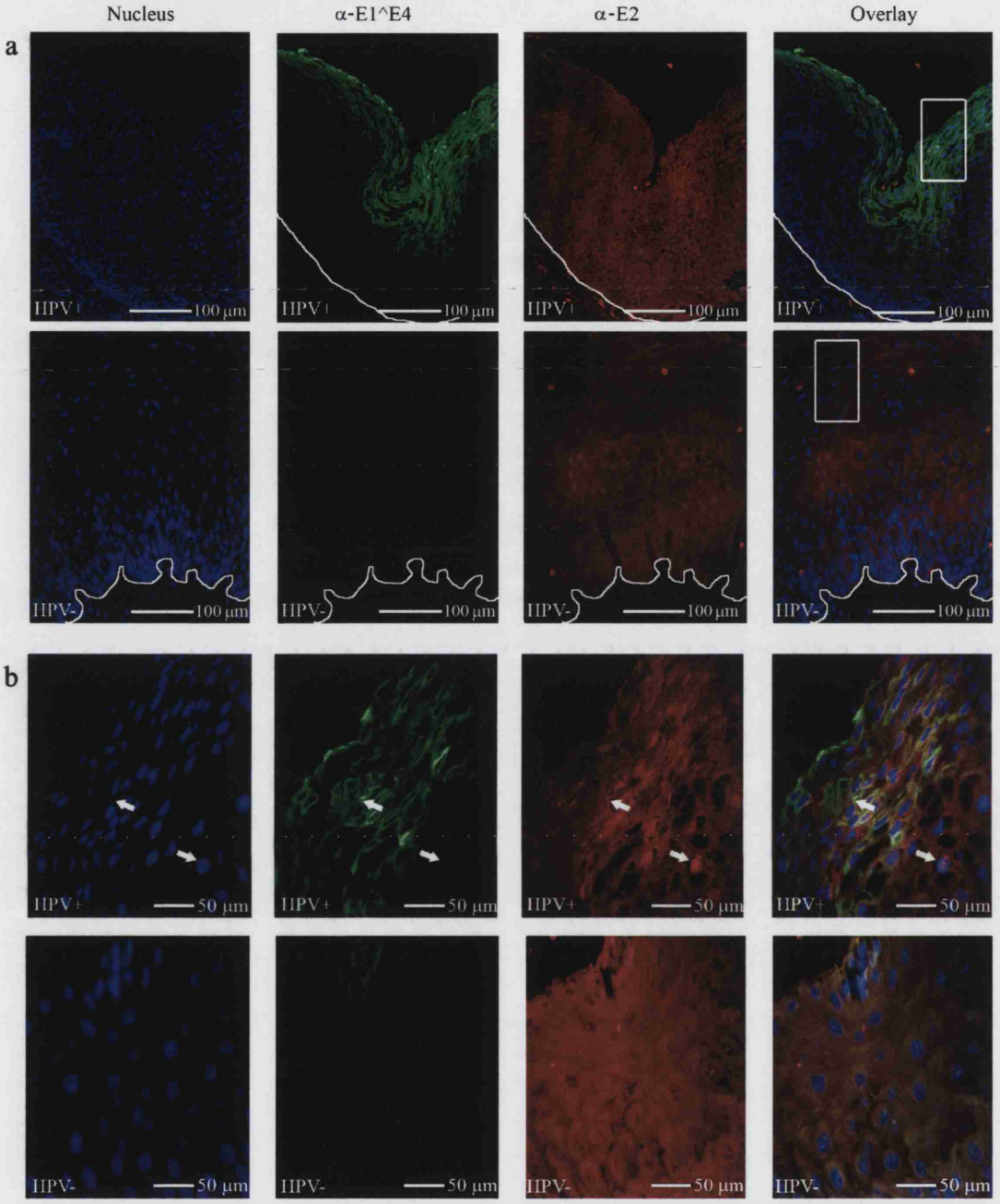


Figure 4.7 E2 and E1^E4 double-stain of a CIN I lesion
(a) E1^E4 (green) was detected using the antibody TVG405 and E2 (red) with the antibody TVG261 in the HPV positive (HPV+) cross-section. The staining pattern of the same E1^E4 and E2 antibodies on a HPV negative (HPV-) part of the lesion is also shown. The nuclei of cells were counterstained DAPI and are shown in blue. The white line marks the position of the basal layer of the epithelium on the low magnification images. The boxed sections are shown at a higher magnification in the same order in (b). Arrows highlight cells which have E2 staining above background levels, where appropriate.

appears to colocalise with E2 in the cytoplasm. Although it is encouraging that these preliminary observations support the *in vitro* evidence for the interaction between E2 and E1^E4, the high background staining of the section due to the E2 antibody makes it difficult to say with certainty that the E2 and E1^E4 proteins actually colocalise *in vivo*. Further optimisation of the staining procedure was required, to try and reduce the high levels of background staining, but due to time limitations it was decided to investigate the functional consequences of the E2/E1^E4 interaction and then if possible return to optimise the *in vivo* staining at a later date.

4.6 Cell-based transcription assay development

Together the experiments described so far in this chapter, suggest that the co-expression of E2 and E1^E4 proteins cause E2 protein accumulation in the cytoplasm. In theory, this could affect E2-mediated transcription, as at low nuclear concentrations, E2 acts as a transcriptional activator and at high nuclear concentrations E2 can act as a transrepressor of the early promoter. To test the effect of E1^E4 on E2-mediated transcription, both proteins were used in cell-based transcription assays with a luciferase reporter plasmid. To perform these experiments without the unnecessary complexity of combining recombinant adenovirus infection with reporter transfection, it was decided to express the E2 and E1^E4 proteins from mammalian expression plasmids instead of from adenoviruses. To ensure that E2 and E1^E4 still interacted when expressed using a different expression system, SiHa cells were co-transfected with pMV11.E2 and pMV11.E1^E4 (gifts from Dr. Kenneth Raj, NIMR, Appendix IV). Cytoplasmic colocalisation between the two proteins was evident using the same staining techniques as used for detecting E2 and E1^E4 proteins following infection with rAd suggesting the interaction was independent of the protein expression system used (Figure 4.8a). C33a cells are derived from an immortalised cervical epithelial cancer and harbour a p53 gene with an inactivating mutation. C33a cells unlike SiHa cells do not contain any HPV DNA sequences or express any HPV proteins. It was important to perform the transcription assays in a HPV null cell line as integrated copies of the HPV promoter could sequester E2 from the reporter plasmid, and the expression of other HPV proteins could make the results of the experiment difficult to interpret given that 16E2 and 16E1^E4 have been shown to associate with several other HPV proteins (Figure 3.11 and 3.12;

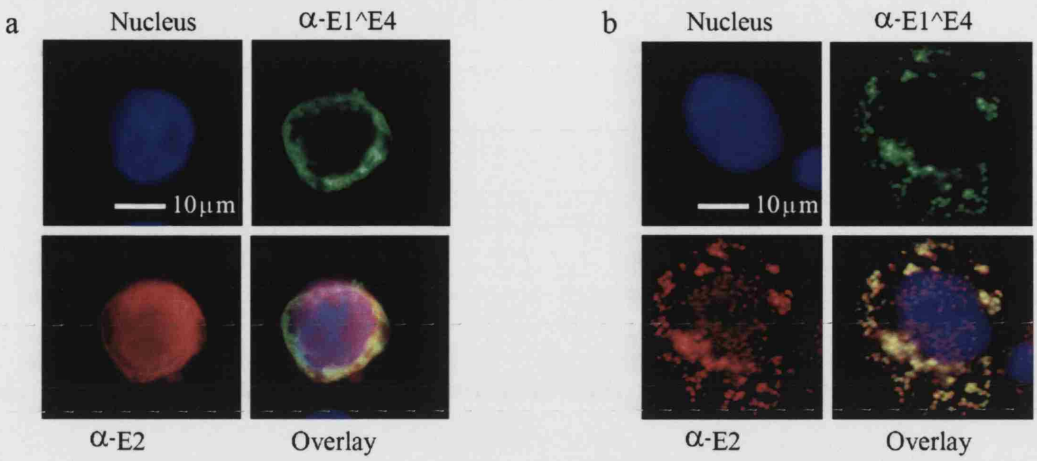


Figure 4.8 Co-expression of E2 and E1^E4 in SiHa and C33 cells
The specified cell types were co-transfected with MV11.E2 and MV11.E1^E4 for 72 h. E1^E4 and E2 were detected by immunofluorescence using the antibodies TVG405 and TVG261, respectively. The nuclei of the cells were counterstained with DAPI. Nuclear (blue), E1^E4 (green) and E2 (red) staining are shown separately and altogether (overlay). (a) SiHa cells. (b) C33a cells.

(Storey et al., 1995; Okoye et al., 2005)). To confirm that the E2 and E1^E4 interaction could also occur in this cell line C33a cells were transfected with E2 and E1^E4 expression vectors. A gain colocalisation of both proteins was found, and a representative image is shown in Figure 4.8b.

The full length HPV16 LCR which incorporates the major early promoter (p97) was cloned into the reporter vector pGL3, upstream of a luciferase reporter gene (Figure 4.9a). This reporter vector should enable both transactivation and transrepression of the early promoter to be estimated by monitoring the activity of the luciferase reporter protein, as the LCR contains four E2 binding sites (BSs) at conserved positions which when occupied can have positive and negative affects on transcription (see Section 1.13.4.1). To test whether the reporter construct created (pGL3.LCR) was functional its activity was compared to that of empty vector (pGL3, Figure 4.9b). It was found that pGL3.LCR displayed luciferase activity which was 10 fold greater than background i.e. pGL3, consistent with the observation that the p97 promoter is constitutively active in the absence of E2 (Hummel et al., 1995; Lewis et al., 1999; Watts et al., 2001). Transfection efficiency was compared in all parallel transcription assays by the co-transfection of the cells with a plasmid that constitutively expressed *Renilla* luciferase. The level of *Renilla* luciferase activity in each sample was monitored and only samples which were transfected with equal efficiencies were compared.

4.7 Effects of E2 on early promoter activity

To establish the effects of different amounts of E2 upon p97 activity, different concentrations of pMV11.E2 were co-transfected with pGL3.LCR in C33a cells. An incremental increase in the amount of pMV11.E2 transfected did not result in a proportional increase in transactivation (Figure 4.10). It was found that E2 had little effect on promoter activity when transfected at a concentration of 2 μg, but only caused weak transactivation of the p97 promoter at a concentration of 4 μg of pMV11.E2, i.e. ~1.6 fold increase over basal promoter activity (Figure 4.10). At concentrations above 4 μg of pMV11.E2 transactivation levels fluctuated around base line (i.e. the basal activity of the promoter in the absence of E2). This is similar to

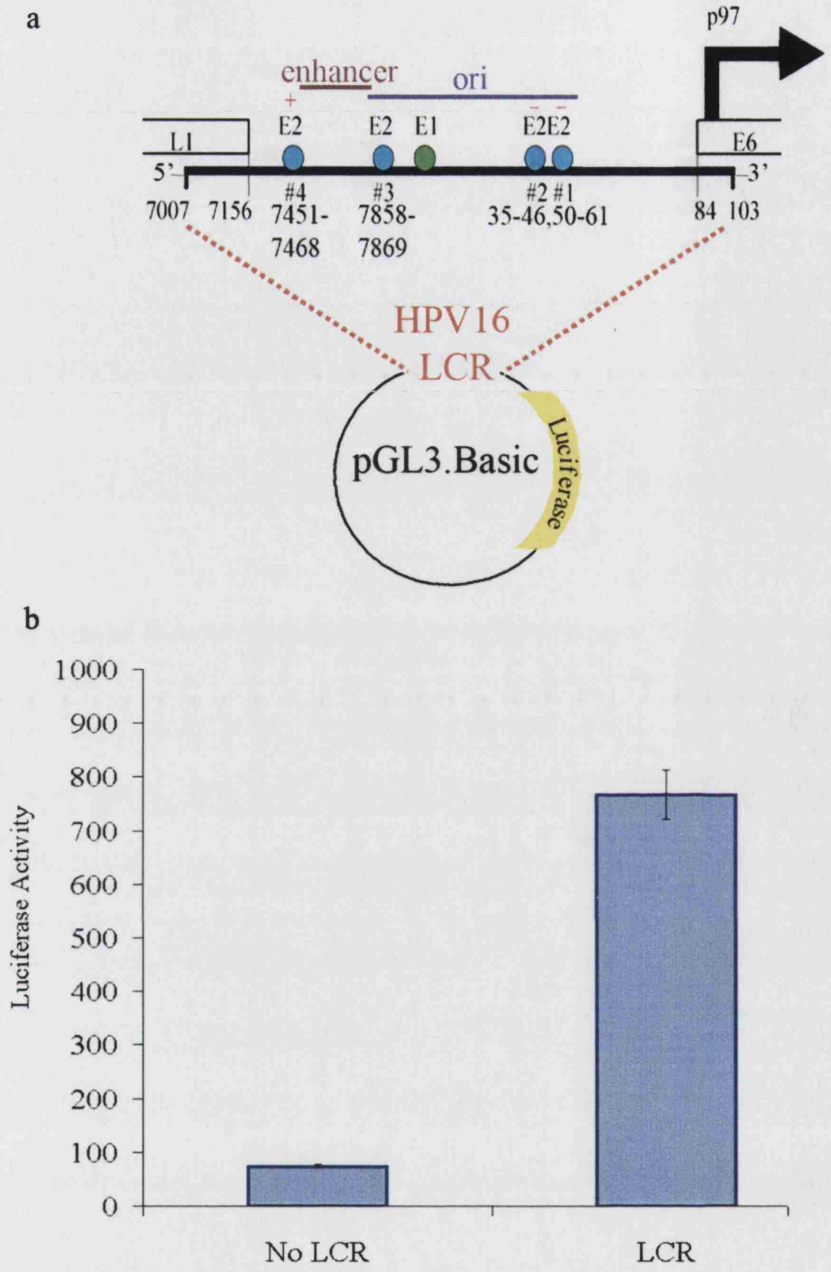


Figure 4.9 Establishing of transcription assays

(a) Schematic of the E2-responsive reporter plasmid (pGL3.LCR), which contains the entire HPV16 LCR and the early promoter (p97) cloned upstream of a luciferase gene. Open boxes represent the HPV ORFs which overlap the LCR and are labelled, coloured circles represent the binding sites for E1 and E2 their nucleotide positions are also specified. The positive and negative symbols shown above three of the E2 BSs indicate the affect of E2 occupancy of these sites on transcription. The relative positions of the viral protein binding sites to the regions of the LCR i.e. origin of replication (ori) and transcriptional enhancer are also shown. (b) C33a cells transfected with pGL3 or pGL3.LCR (1 μg), pMV11 (13 μg) and phRL-TK (1 μg). Transfected cells were assayed for luciferase activity. The error bars represent the SE of three independent samples.

what others have found in analogous experiments, and is consistent with the hypothesis that at low E2 concentrations, the promoter distal E2 binding site (BS#4) is occupied activating the promoter, and at high E2 concentrations in addition to BS#4 the promoter proximal E2 binding sites (BS#1 and #2) are also occupied counteracting promoter activitation (Figure 4.10) (Romanczuk et al., 1990; Thierry and Howley, 1991; Tan et al., 1992; Sanders and Maitland, 1994; Demeret et al., 1997; Alloul and Sherman, 1999; Kim et al., 2003b).

4.8 Effect of E1^E4 on E2-mediated transcription

Immunofluorescence experiments showed that E2 and E1^E4 protein colocalisation occurred more frequently after 48 h and 72 h of co-expression than after 24 h. To be able to test the effect of E2/E1^E4 colocalisation on the activity of the early promoter transcription assays were performed at 24 h, 48 h and 72 h post-transfection. E1^E4 alone was shown to approximately halve the level of basal transcription by the LCR at all the time points examined (Figure 4.11a). The co-expression of E2 and E1^E4 with the reporter plasmid reduced the repressive effect of E1^E4 slightly at all time points, but together successfully repressed basal transcription (Figure 4.11a).

The co-expression of E2 and E1^E4 reduced the level of E1^E4 repression, but transcription from the p97 promoter was still inhibited. However, the repressive effect of E1^E4 did not seem to correlate with the frequency of E2/E1^E4 colocalisation, but instead it appeared to change with E2 levels, i.e. the repressive effect of E1^E4 on E2-mediated transcription was the least when E2 levels were the highest, although repression was still not relieved (48 h post transfection, Figure 4.11b).

4.9 The effect of E1 on E2-mediated transcription

E1 had been previously shown to increase E2-mediated transcription in similar assays (Piccini et al., 1995; Demeret et al., 1998). The E1 protein is thought to be co-expressed with E2 and E1^E4 *in vivo*, and for this reason it seemed important to express E1 with E2 and E1^E4 in similar experiments. Transcription assays were performed following transfection of equal amounts of pMV11.E1 and pMV11.E2 with

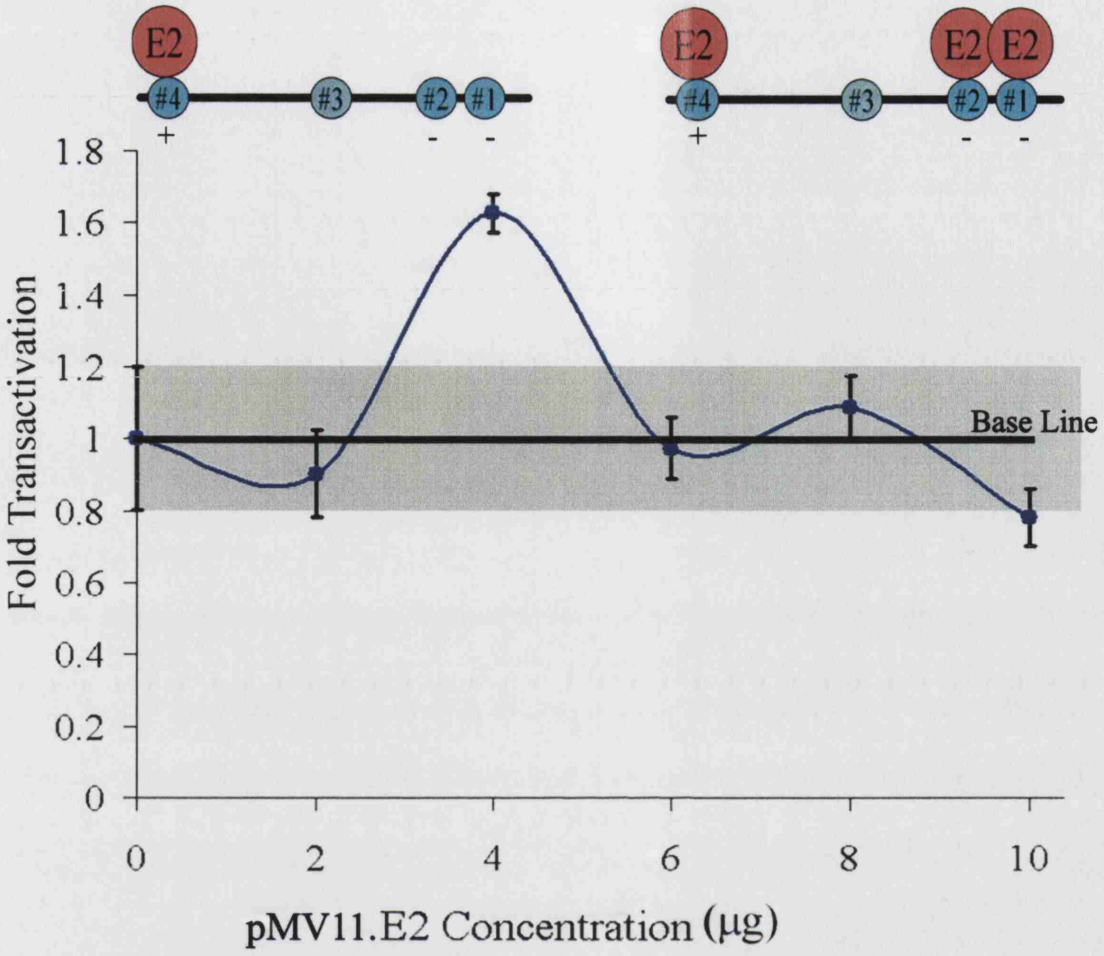
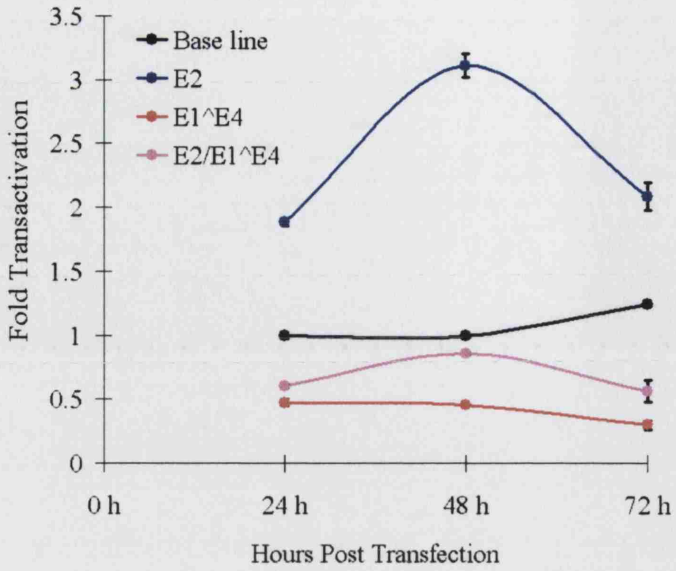


Figure 4.10 Effect of increasing E2 concentration on basal transcription from the LCR reporter plasmid

C33a cells were transfected with 2-10 μg of pMV11.E2, 1 μg of pGL3.LCR and 1 μg phRL-TK for 24 h (NB transfections made up to 15 μg of DNA with pMV11). The samples were then assayed for luciferase activity, and total protein concentration. The graph shows the fold transactivation of each sample with respect to the basal promoter activity. This was calculated by dividing the luciferase activity for 120-130 μg of each cell extract by that for pGL3.LCR (1 μg), phRL-TK (1 μg) and pMV11 (13 μg) transfected cells. The error bars shown on the graph represent the SE of three independent samples, and the grey shading represents the base line error. Above the graph schematic representations of the LCR is shown. The E2 binding sites that are proposed to be filled at low (left) and high E2 (right) concentrations are shown.

a



b

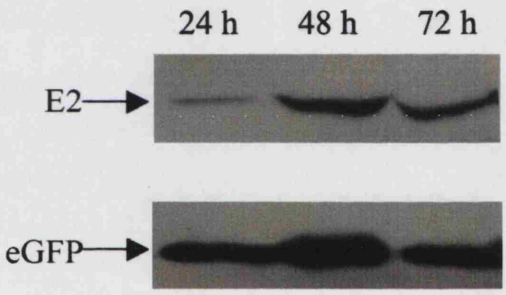


Figure 4.11 Effect of E1^E4, on E2-mediated transcription

(a) C33a cells were transfected with 1 μg of pGL3.LCR, 2.5 μg of pMV11.E2, 1 μg phRL-TK and/or pMV11.E1^E4, for 24 h, 48 h and 72 h (NB transfections made up to 15 μg of DNA with pMV11). The samples were then assayed for luciferase activity, and total protein concentration. The graph shows Log10 fold transactivation of each sample with respect to the basal promoter activity. This was calculated by dividing the luciferase activity for 120-130 μg of each cell extract by that for pGL3.LCR (1 μg), phRL-TK (1 μg) and pMV11 (13 μg) transfected cells. The error bars shown on the graph represent the SE of three independent samples. (b) C33a cells were transfected with 1 μg of pIRES2eGFP (as a transfection control) and 2.5 μg of pMV11.E2 for 24 h, 48 h and 72 h (NB transfections made up to 15 μg of DNA with pMV11). The samples were then Western blotted with E2 (TVG261) and eGFP (sc-9996) antibodies.

the reporter plasmid (pMV11.E1 was a kind gift from Dr.Ken Raj, NIMR). In these experiments E1 alone had little effect on p97 activity in the absence of E2, but a progressive increase in luciferase activity was observed from 24 h to 72 h post transfection (Figure 4.12). At 72 h post transfection, E1/E2 transactivation of the early promoter was 19 fold above the affect of E2 alone on the promoter, which is similar to the 16 fold increase reported by Demeret *et al* in HPV18 transactivation studies (Demeret et al., 1998). It was decided to study the effect of E1^E4 on E1/E2-mediated transcription at 72 h post transfection as at this time point transactivation of the p97 promoter was shown to be the highest by E1 and E2 proteins, and also because E2/E1^E4 colocalisation was found to occur more frequently at later time points than earlier ones.

4.10 Comparison of the effects of different amounts of E1 and E2 upon p97 activity

Transient expression levels of proteins vary with transfection time, as does the effect of E1 and E2 on transcription (Figure 4.10 and 4.11b). This is consistent with the findings of others that the ratio and/or levels of protein co-expression influence transactivation levels (discussed in Section 1.13.3.1 and 1.13.4.1), (Sandler et al., 1993; Le Moal et al., 1994; Sanders and Maitland, 1994; Stubenrauch et al., 1996; Ferran and McBride, 1998).

The effect of different concentrations of E1 and E2 expression vectors on transcription was tested. It was found that at all the concentrations tested E1 increased E2-mediated transcription. However, at low E1 concentrations (0.5 μg) the transactivational effect of E1 on the p97 promoter did not change much with E2 concentration (Figure 4.13a). At concentrations of pMV11.E1 >0.5 μg, the magnitude of the positive affect of E1 on transcription fluctuated with the amount of E2. It was found that at pMV11.E2 concentrations of ≤6 μg the addition of either 1 or 2 μg of pMV11.E1 had a similar affect on transactivation. However, at pMV11.E2 concentrations that were >6 μg, the addition of 1 μg of pMV11.E1 caused the level of transactivation to decline, whereas the addition of 2 μg of E1 allowed the level of transactivation to continue to increase.

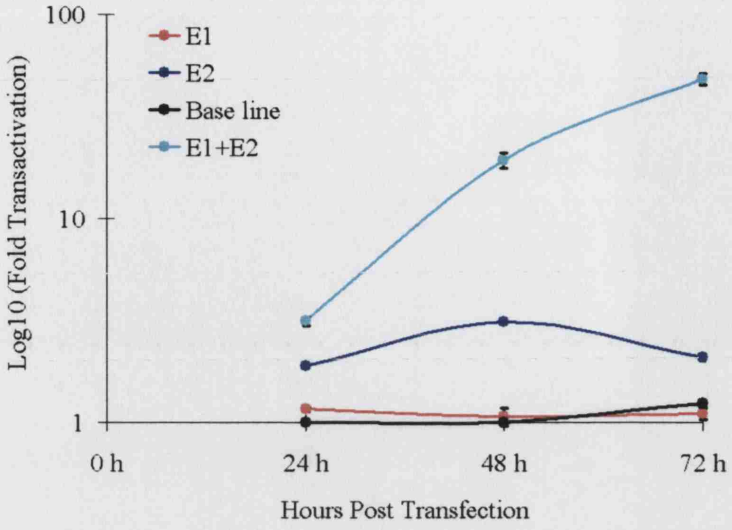
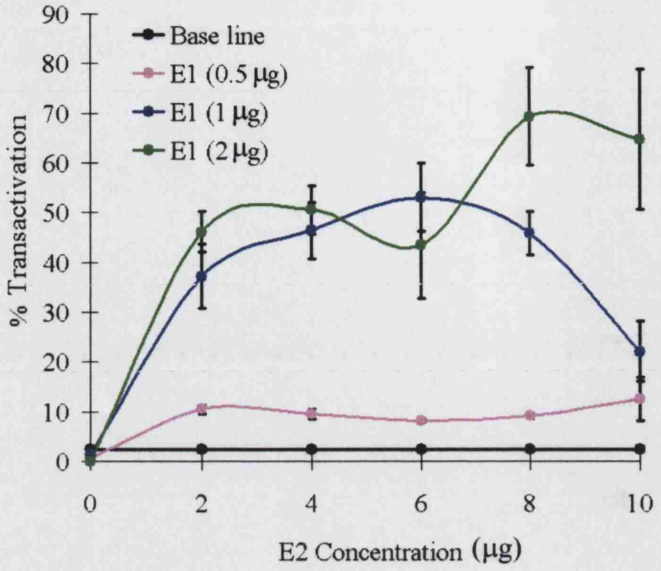


Figure 4.12 Effect of E1 on E2-mediated transcription
C33a cells were transfected with 1 μg of pGL3.LCR, 1 μg phRL-TK and 2.5 μg of pMV11.E2 and pMV11.E1 as specified, for 24 h, 48 h and 72 h (NB transfections made up to 15 μg of DNA with pMV11). The samples were then assayed for luciferase activity, and total protein concentration. The graph shows the fold transactivation of each sample with respect to the basal promoter activity. This was calculated by dividing the luciferase activity for 120-130 μg of each cell extract by that for pGL3.LCR (1 μg), phRL-TK (1μg) and pMV11 (13 μg) transfected cells. The error bars shown on the graph represent the SE of three independent samples.

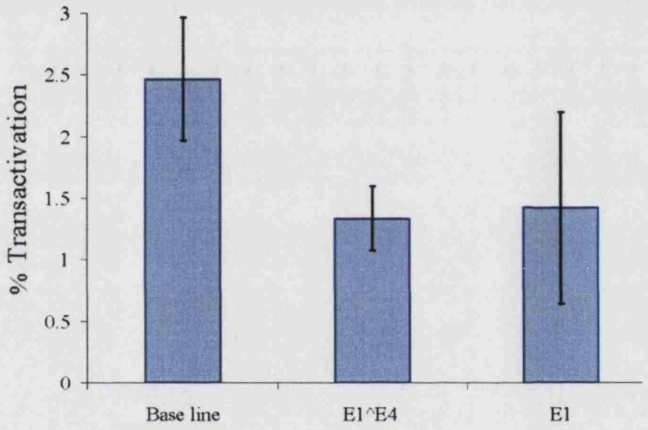
Figure 4.13 Effect of E1 and E1^E4 on E2-mediated transcription

C33a cells were transfected with 1 μg of pGL3.LCR, 1 μg phRL-TK and pMV11.E2, pMV11.E1 and pMV11.E1^E4 as indicated, for 72 h (NB transfections made up to 18 μg of DNA with pMV11). The samples were then assayed for luciferase activity, and total protein concentration. The graph shows the percentage transactivation of each sample with respect to the basal promoter activity. This was calculated by subtracting the luciferase activity for the mean of 120-130 μg of pGL3.LCR (1 μg), phRL-TK (1μg) and pMV11 (16 μg) transfected cells from that of each cell extract and then converting this value to a percentage, where the highest value in each experiment was taken as a 100 %. The error bars shown on the graph represent the SE of at least six independent samples. (a) Graph to show the effect of different amounts of E1 and E2 on p97 activity. (b) Bar chart to show the effect of E1^E4 (1 μg), and E1 (1 μg) on basal transcription from the LCR (c) Graph to show the effect of E1^E4 (1 μg), and E1 (1 μg) on E2 (2-10 μg) -mediated transcription from the p97 promoter.

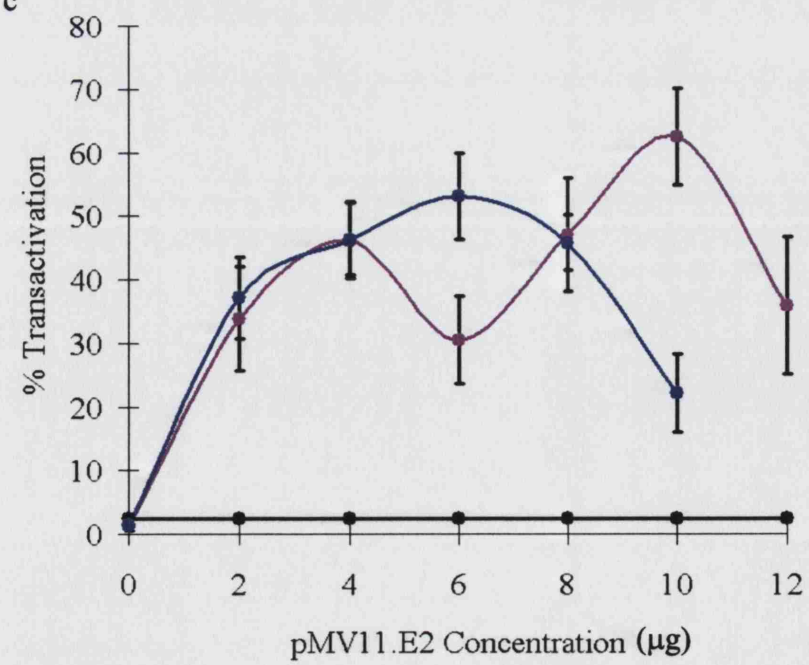
a


Base line
E1 (0.5 µg)
E1 (1 µg)
E1 (2 µg)
% Transactivation
E2 Concentration (µg)

b


% Transactivation
Base line
E1^E4
E1

c


% Transactivation
pMV11.E2 Concentration (µg)

These experiments show that at the ratio of E1 to E2 of 1:1-1:6 the level of transactivation continues to increase, however, when the ratio of E1 to E2 is increased further i.e. 1:8-1:10, a decrease in the amplitude of transactivation is seen (Figure 4.13a). This suggests that the ratio of E1 to E2, as well as the levels of the two proteins may be important in regulating early promoter activity.

4.11 The effect of E1^E4 on E1/E2-mediated transactivation

A range of pMV11.E2 concentrations with a stable pMV11.E1 concentration (1 μg) ±pMV11.E1^E4 (1 μg), were used in transcription assays to test the effect of E1^E4 on the transcriptional activity of E2. It was decided to use a stable pMV11.E1 concentration (1 μg) in these experiments, as at this concentration of pMV11.E1 the magnitude of E2-mediated transactivation varied with E2 levels, thus potentially an increase or a decrease in transcription activity by E1^E4 could be seen at different pMV11.E2 concentrations. E1^E4 was still found to have a small repressive effect on transcription from the p97 promoter in the presence of E1 but in the absence of E2 (Figure 4.13b). E1 also caused a decrease in basal transcription in the absence of other viral proteins but the data suggests that the decrease is not significant (Figure 4.13b).

At the transfection ratio of 2 to 1, or 4 to 1, of E2 to E1^E4, E1^E4 was found to have little effect on E1/E2-mediated transactivation (Figure 4.13c). However, at the point where E1/E2-mediated transactivation was optimal in the experiment i.e. a transfection ratio of E1 to E2 of 1 to 6, E1^E4 caused a reduction in transactivion by approximately 20 %, to within the range that is induced by a transfection ratio of E1 to E2 of 1 to 2 (Figure 4.13c). This suggests that E1^E4 may be able to perturb the optimal transactivation ratio of E1 to E2, by possibly reducing nuclear E2 levels. This theory is supported by the finding that at ratios of E2 to E1^E4 that are higher than 6 to 1 the level of transactivation mimics the effect of E1 and E2 alone on transactivation but for lower E2 concentrations i.e. E2 to E1^E4 concentrations of 8 to 1, 10 to 1 and 12 to 1 give levels of transactivation that are equivalent to E1 to E2 concentrations of 1 to 4, 1 to 6 and 1 to 8. This is consistent with the hypothesis that E1^E4 may effect nuclear E2 levels, as at high transfection ratios of E2 to E1^E4 expression vectors. The data suggests that the sequestration of E2 in the cytoplasm by

E1^E4 may be able to shift the nuclear E1 to E2 transactivation profile to the left, and in this way allow E1/E2-mediated transactivation to continue at high levels of E2, which would in the absence of E1^E4 inhibit transactivation.

These experiments show that E1^E4 can either have no effect, a positive effect or a negative effect on p97 promoter activation levels when co-expressed with E1 and E2, depending on the levels at which E1^E4 is co-expressed with the two proteins. To test the contribution of E1^E4-mediated G2 arrest on E1/E2-mediated transcription, a double E1^E4 mutant (T22A and T23A), that is defective in its ability to cause G2 arrest was used in transcription assays (pMV11.T22A, T23A 16E1^E4 was kindly provided by Dr. Clare Davy, NIMR; (Davy et al., 2002). The G2 arrest-defective E1^E4 mutant was shown to be expressed at similar levels to wild-type (WT) E1^E4 in these assays and also to give a similar transactivation profile to WT E1^E4 (Figure 4.14a and b). This result implies the G2 arrest function of E1^E4 does not affect the p97 promoter. Coincidently, like WT E1^E4, the G2 arrest defective E1^E4 mutant was still able to repress basal transcription activity in the absence of E1 or E2 proteins.

4.12 Discussion

E1^E4 is a cytoplasmic protein that colocalises with keratins and mitochondria. In contrast, E2 is a predominantly nuclear protein but is sometimes detected in the cytoplasm of cells. It has been suggested that cytoplasmic E2 is an artefact of E2 over expression from recombinant expression vectors (Penrose and McBride, 2000). However, the identification of cytoplasmic 16E2 in W12E cells and low grade HPV lesions challenges this view (Maitland et al., 1998; Bechtold et al., 2003). All the known roles of E2 in the HPV life cycle require E2 to be present in the nucleus, but this does not mean that the cytoplasmic retention of E2 does not affect known E2 functions. In fact several cellular transcription and replication factors cycle between the nucleus and the cytoplasm, and cytoplasmic retention of these factors appears simply to be a means of modulating their function. An example of a cycling cellular TF is the interferon regulatory factor 3 (IRF-3) which activates the transcription of the interferon β gene (IFN-β). IRF-3 is retained in the cytoplasm unless activated by phosphorylation, which occurs in response to viral infection (Mori et al., 2004). Similar regulation has also been reported for cellular replication factors, and indeed,

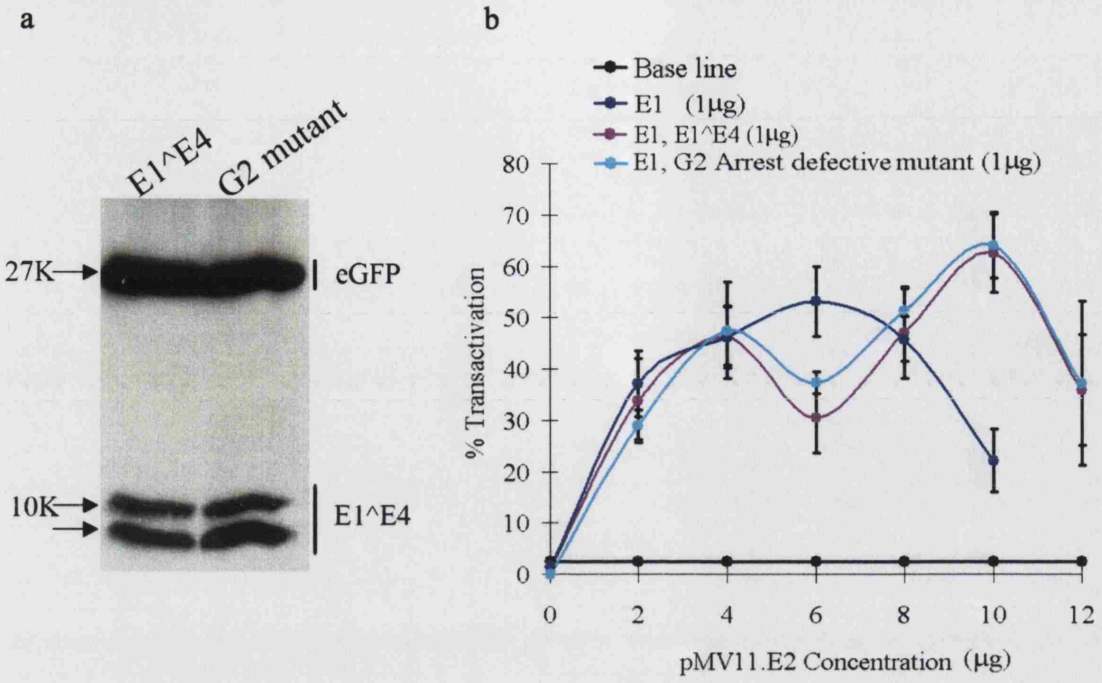


Figure 4.14 Effect of E1^E4 and the G2 arrest defective E1^E4 mutant on E1/E2-mediated transcription

(a) C33a cells were transfected with 1 μg of pGL3.LCR, 1 μg phRL-TK and pMV11.E2, pMV11.E1, pMV11.E1^E4 and pMV11.T22A,T23A E1^E4 (G2 arrest defective mutant) as indicated, for 72 h (NB transfections made up to 18 μg of DNA with pMV11). The samples were then assayed for luciferase activity, and total protein concentration. The graph shows the percentage transactivation of each sample with respect to the basal promoter activity. This was calculated by subtracting the mean luciferase activity for 120-130 μg of pGL3.LCR (1 μg), phRL-TK (1μg) and pMV11 (16 μg) transfected cells from that of each cell extract and then converting this value to a percentage, where the highest value in each experiment was taken as a 100 %. The error bars shown on the graph represent the SE of at least six independent samples. (b) C33a cells were co-transfected with pIRESeGFP and pMV11.E1^E4 or pMV11.T22A,T23A E1^E4 were Western blotted for E1^E4 (TVG402) and eGFP. The two E1^E4 bands seen correspond to the unphosphorylated (10K) and the N-terminally cleaved (8K) forms of E1^E4.

the replication initiation protein cdc6 behaves in this way (Dutta and Bell, 1997). It is noteworthy that the role of E2 in the initiation of viral replication is functionally analogous to that of cdc6 in cellular replication, as both these proteins act as loading factors that facilitate the formation of active helicases at origins of DNA replication (Dutta and Bell, 1997; Sclafani et al., 2004).

The data suggests that E1^E4 by modulating nuclear E2 levels can affect the level of activation of the early promoter. In addition it is possible that the cytoplasmic sequestration of E2 by E1^E4 may also serve as a mechanism of preserving a pool of functional E2 in cells. It is tempting to speculate that the complete cytoplasmic sequestration of E2 from the nucleus by E1^E4 observed in 5 % of cells, occurred only transiently, and that subsequently some if not all the cytoplasmic E2 would have been returned to the nucleus enabling E2 to activate the p97 promoter.

E1^E4 was shown to cause cytoplasmic E2 to increase with time, suggesting the interaction between E2 and E1^E4 could play a role in modulating the levels of nuclear E2. In both SiHa and C33a cells, cytoplasmic E2 was found to colocalise with E1^E4 bound to keratins or mitochondria, although the latter was seen more frequently. It is not clear if the accumulation of E2 at mitochondria is of functional significance, or if it is just circumstantial (i.e. it was observed more frequently just because E2/E1^E4 colocalisation is more common at time points when E1^E4 is usually found to be associated with mitochondria). In support of the former possibility, SiHa cells co-expressing both E2 and E1^E4 proteins from rAd vectors, displayed a higher tendency to undergo apoptotic cell death at 48 h and 72 h, than at 24 h post infection, in comparison with cells which just express either E2 or E1^E4 (Dr. Qian Wang, NIMR, *Personal communication*). Both E2 and E1^E4 proteins have been implicated as apoptosis promoters in the literature, and it would be interesting to determine whether the accumulation of E2 and E1^E4 at mitochondria directly contributes to apoptosis (Sanchez-Perez et al., 1997; Desaintes et al., 1999; Webster et al., 2000; Raj et al., 2004).

To provide *in vivo* evidence for the E2/E1^E4 interactions, immunohistological analysis of low grade HPV16 cervical epithelial lesions was attempted. These experiments in agreement with previous studies, clearly showed that 16E1^E4 was

abundantly expressed in the cytoplasm of cells found in the upper layers of infected epithelium (Figure 4.7a), (Doorbar et al., 1997; Peh et al., 2002; Middleton, 2003). However, the detection of 16E2 in HPV lesions proved to be a difficult task, despite the use of the same E2 antibody that was used successfully by Maitland *et al* to identify E2 in low-grade lesions caused by HPV16 (Maitland et al., 1998; Stevenson et al., 2000). It is possible that the difference in HPV16 lesion-staining in this study compared to that produced by Maitland *et al*, may be due to the fact that in the latter, the antibody was affinity-purified but was not before use in our experiments. Affinity-purification of antibody containing serum is thought to reduce non-specific background staining, and may explain why clear specific E2 staining was not achieved using this antibody in our hands.

There is however little doubt that E2 is expressed in the same cells as E1^E4 following the onset of genome amplification. The E2 protein is essential for vegetative viral DNA replication, and several reports have observed that the expression of E1^E4 coincides with the amplification of viral episomes in the upper epithelial layers (Breitburd et al., 1987; Doorbar et al., 1997; Peh et al., 2002). Although it remains the subject of speculation whether an association between E2 and E1^E4 occurs in HPV induced lesions, 16E2 has been identified in the upper epithelial layers of low-grade lesions, revealing a distribution similar to that seen using antibodies to 16E1^E4 by Doorbar *et al* in a different study (Doorbar et al., 1997; Maitland et al., 1998).

E1^E4 was found to alone and in combination with E2 to reproducibly repress the basal activity of the early promoter. The repressive effect of E1^E4 on basal promoter activity is unlikely to be mediated by a direct interaction with the LCR, as E1^E4 is predominantly a cytoplasmic protein that has been shown to have no affinity for DNA (Doorbar et al., 2000). It is possible that E1^E4 in the absence of E2 may sequester factors required for basal transcription of the p97 promoter in the cytoplasm and in this way inhibit transcription. This is supported by the finding that E1^E4 can associate with the cellular DNA binding protein toperisomerase II β-BP1 (TopBP1), an enhancer of E2-mediated transcription (Doorbar, ; Doorbar et al., 2000; Boner et al., 2002). Although, E1^E4-mediated G2 arrest could also be responsible for the effect of E1^E4 alone on p97 activity. This repressive effect of E1^E4 on the early

promoter may be important in the modulation of early gene transcription in the early stages of the HPV life cycle.

In contrast, the effect of E1^E4 on E1/E2-mediated transcription resulted in the transactivation of the early promoter to various levels depending on the levels of the three proteins. The positive effect of E1 on transcription was dependent on the presence of E2, whereas the effect of E1^E4 on transcription was dependent on the expression of E1 and E2. Together this suggests that the transactivational effect of both these proteins on the LCR is mediated via E2.

The association between E1 and E2 causes a conformational change in the N-terminal transactivation domain (TAD) of E2 which it is thought may facilitate transcription (Ferguson and Botchan, 1996; Parker et al., 2000). In addition, E1 may also be able to act as a co-transcription factor, as it is too a transcription factor in its own right (Demeret et al., 1998). The magnitude of the transactivional effect of E1 on E2 was dependent on both the levels and the ratios of the two proteins. The differential effects of E1 that were observed when E2 was co-expressed at high or low concentrations could be due to the loss of balance between the two proteins. This may cause an increase in free E1 or E2 molecules in the cell which could sequester cellular TFs (that act on the LCR), away from the promoter. Alternatively, high E2 to E1 ratios could inhibit E1/E2-mediated transcription by the formation of high levels of E2/E2 complexes which may compete with E1/E2 complexes for E2 BS #3 occupancy.

It is hypothesised that E1^E4 modulates E1/E2-mediated transcription by regulating nuclear E2 concentration. In this way, E1^E4 may fine tune E1/E2-mediated early gene transcription during the productive stage of the HPV life cycle. It seems appropriate to correlate the effect of E1, E2 and E1^E4 on the early promoter to the genome amplification stage of the virus life cycle, as this is when E1, E2 and E1^E4 are expressed together at high levels (Higgins et al., 1992; Klumpp and Laimins, 1999). In addition it has been shown that in undifferentiated cells that the E2 BS #3 is frequently hypermethylated. This is not thought to be the case in differentiated cells (Kim et al., 2003b; Kalantari et al., 2004).

In the basal and parabasal layers early gene transcription is thought to be regulated by the concentration-dependent filling of the promoter distal and proximal E2 binding sites, and also by the chromatin structure of the LCR (Bechtold et al., 2003; Kim et al., 2003b). This suggests that the repressive effect of E1^E4 on the basal activity of the p97 promoter may be important during the early stages of the virus life cycle, when the ability of E2 to repress the p97 promoter is thought to be prevented by the chromatin structure of the LCR (Bechtold et al., 2003).

The transactivational effect of E1^E4 may apply during the genome amplification phase of the virus life cycle. This fits with the current understanding that transcription from the early promoter is regulated in different ways in the upper layers of the epithelium to the lower layers (Kim et al., 2003b). The mechanism proposed to explain the effect of E1^E4 on E1/E2-mediated transcription is that the cytoplasmic retention of E2 by E1^E4 may be able to change the nuclear ratio of E1 to E2, by reducing levels of E2 in the nucleus. In support of this a qualitative difference in nuclear E2 in E1^E4 expressing cells were observed by immunofluorescence.

The use of a G2 arrest defective E1^E4 mutant in transcription assays eliminated the role of E1^E4 induced cell cycle arrest on transcription, at least under the experimental conditions that were used. However, more evidence is required to be able to say definitively that the effect of E1^E4 on E1/E2-mediated transcription is due to the direct association between E2 and E1^E4. It is noteworthy that the effects of E1^E4 on transcription in these assays are likely to be mediated by full length E1^E4 and the N-terminally cleaved form of E1^E4, but not by the phosphorylated form of E1^E4, as the latter was not detected in C33a cells.

Together these experiments show that changing the ratios and levels of E1, E2 and E1^E4 proteins in the context of each other can cause fluctuations in transcription from the early promoter. Although, these differences could in part be related to differences in the levels of replication of the LCR-containing reporter plasmid. The ratios of E1, E2 and E1^E4 that were used in these experiments were chosen methodically but this does not mean they bear any resemblance on the ratios of the proteins that are translated from polycistronic mRNA transcripts encoded by the HPV16 late promoter (p670). For this reason it seemed important to test the effect of

E1, E2 and E1^E4 on the transcriptional activity and replication of the LCR-containing reporter plasmid following the co-expression of these proteins at ratios that mimicked those found *in vivo*.

Chapter 5 E1^E4 enhances E1/E2-mediated transcription and ori-dependent replication

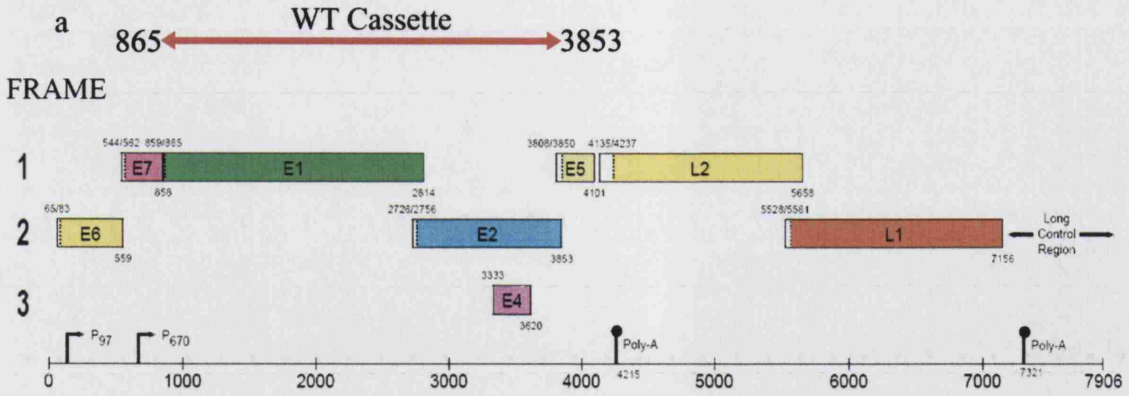
5.1 Introduction

The expression of the E1^E4 protein is linked to that of E1 through its shared initiation codon, and to E2 by the position of E4 within the E2 ORF. Transcripts that encode E1, E2 and E1^E4 are produced throughout the virus life cycle (Durst et al., 1992a; Higgins et al., 1992; Ozbun and Meyers, 1998b). However with the activation of the differentiation-dependent promoter, p670 (in HPV16), transcription of polycistronic mRNAs that encode E1, E2 and E1^E4 spliced to the E5 ORF are upregulated (Klumpp and Laimins, 1999). The levels of the different early region transcripts produced from p670 are thought to be regulated by the strength of the splice donor and splice acceptor sites within the transcriptional unit expressed from p670 (Grassmann et al., 1996; Remm et al., 1999; Wang et al., 2003). The presence of a strong splice donor site at the beginning of the E1 ORF means that the most abundant p670 transcript encodes the E1^E4 protein and correspondingly the least abundant transcript produced by p670 encodes E1 (Remm et al., 1999).

In order to study the effects of E1, E2 and E1^E4 proteins expressed at ratios that mimicked expression in the late stages of the virus life cycle, it was decided to use an E1/E2/E4 cassette system in cell-based functional assays. The E1/E2/E4 cassette was cloned into a mammalian expression vector downstream of a CMV promoter. It was hoped that the transcription patterns from this cassette would be analogous to mRNAs transcribed from the late promoter *in vivo*.

5.2 The production of a WT and mutant E1/E2/E4 cassette

The E1/E2/E4 ORFs were originally amplified directly from the HPV16 genome by PCR and were then sub-cloned into a bacterial expression vector (Figure 5.1a). The cassette sequence was then subjected to site-directed mutagenesis using specific primers which introduced two base pair mutations in the E2 and E4 ORFs. These thymine (T) to adenine (A) mutations converted codons 15 and 16 (encoding leucines) of E1^E4 to stop codons, without effecting the coding potential of the overlapping



b

GCAGCAACGAAGTATCCTCTCCTGAAATTATTA
A A

E1^E4 A_6 A T K Y P L L K_{14} STOP STOP

AGCAACGAAGTATCCTCTCCTGAAATTATTA
A A

E2 S_{202} N E V S S P E I I_{211}

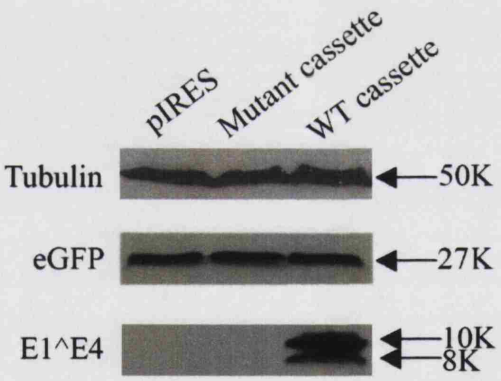


Figure 5.1 WT and mutant cassette production

(a) Linear representation of the HPV16 genome (Adapted from Baker and Calef, 1996). The rectangles represent the major viral open reading frames (ORFs) as labelled. The first nucleotide number above each box corresponds to the start of the ORF and the number shown below each box is the position of the end of the ORF. The coloured part of the rectangles corresponds to the position of the coding sequences within each ORF. The second nucleotide number above each box and the dotted line within the box represent the position of the start codon within each ORF. Below the ORFs is a scale of the genome in bp. The positions of promoters (arrowed) and polyadenylation sequences are also shown. The red arrow specifies the region of the HPV16 genome that was cloned into the pIRES2eGFP vector (pIRES). (b) Coding sequence of the first 11 amino acids of the E4 ORF, which is spliced to the first 5 amino acids of the E1 ORF to form E1^E4 (not shown) and the corresponding coding sequence within the overlapping E2 ORF. The base changes that were induced by site-directed mutagenesis in this region of the WT cassette to produce the mutant cassette are indicated in red. The mutations created two stop codons in the E4 ORF but only caused silent changes in the E2 reading frame. (c) Anti-tubulin and anti-E1^E4 (TVG402) Western blots of total cell extracts following transfection of C33a cells for 48 h with plasmid expression vectors as labelled.

E2 ORF (Figure 5.1b, the sub-cloning of the cassette and the site-directed mutagenesis was performed by Dr. Woei Ling Peh, NIMR (Peh, 2002)). The E1/E2/E4 wild-type (WT) cassette and the E1^E4 knock-out (mutant) cassettes were then inserted (via the *EcoRI* and *BamHI* restriction sites) into the multiple cloning site (MCS) of the mammalian expression vector pIRES2.eGFP (pIRES; by Dr. Heather Griffin, NIMR). The mutant cassette was produced, to serve as a negative control. The pIRES vector contains the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) between the MCS and the enhanced green fluorescent protein (eGFP) gene, and permitted the viral ORFs and the eGFP gene to be translated from polycistronic mRNAs. This means that either E1, E2 or E1^E4 would be the first ORFs present on each spliced transcript, which is similar to the transcripts produced from the p670 promoter.

In functional assays the pIRES.mutant cassette (mutant cassette) and the pIRES.WT cassette (WT cassette) were transfected in parallel to allow the effect of E1^E4, E1 and E2, to be separated from that of E1 and E2 alone. To confirm that E1^E4 protein was produced only by the WT cassette, both cassette constructs were transfected into C33a cells and the cell extracts were subsequently Western blotted. It was found that despite comparable extract loading and eGFP expression between WT and mutant samples, E1^E4 protein was only detected in the former, suggesting that the stop codons introduced in the E4 ORF successfully prevented the expression of E1^E4 from the mutant cassette (Figure 5.1c). The two forms of E1^E4 that were detected in the WT cassette sample were the full-length and the N-terminally cleaved form of E1^E4 but not the phosphorylated form of E1^E4 (Figure 5.1c). This was expected as it was shown in the previous chapter that following E1^E4 expression in C33a cells, the phosphorylated form of E1^E4 was not identified (Figure 4.14a).

5.3 Assessing the effect of the mutations in the E2 and E4 ORFs on splicing patterns from transcripts produced from the mutant cassettes

The mutations introduced into the WT cassette to produce the mutant cassette were at nucleotide positions (nt) 3383 and 3386. These sites are respectively 25 and 28 bps away from the splice acceptor junction for E4 at nt 3358, which can pair with the

strong splice donor site within the E1 ORF at nt 880 (Figure 5.2b), (Grassmann et al., 1996). RNA splicing is dependent on the secondary structure of the RNA within the vicinity of the splice junction (reviewed in (Buratti and Baralle, 2004)). To test whether these mutations caused changes in splicing patterns, total RNA was extracted from WT and mutant cassette transfected cells and analysed by Northern blotting (Figure 5.2b).

Ribosomal RNA 28S and 18S subunits were found to be abundant in the RNA extracts, and were used as internal markers to estimate the sizes of the RNA bands. Two bands were detected in the WT and mutant cassette samples using a radioactively labelled probe that specifically recognised E1, E2 and E4 ORFs but not cellular transcripts, or transcripts produced from the pIRES back-bone (Figure 5.2b). The bands that were detected in the WT and mutant cassette extracts were of approximately equal intensity, suggesting that they were produced at equal levels by the two systems. It was predicted on the basis of their sizes that the two bands identified using a probe that recognised E1, E2 and E4 ORFs were the unspliced mRNA (4.57 kb) and the E1^E4 transcript (2.092 kb, Figure 5.2a and b), (Baker and Calef, 1996).

The other transcripts predicted to be produced by the cassettes were not detected in either the WT or mutant cassette samples by Northern blotting, possibly because their abundance in the extracts was low in comparison with the unspliced and the E1^E4 transcripts. This result is consistent with findings by Doorbar *et al* following the analysis of early transcripts produced in W12E cells (Figure 5.2a), (Doorbar et al., 1990). The results of the Northern blot do however suggest that the mutations introduced into the cassette do not affect the splicing of the E1^E4 transcript. Reverse-transcriptase PCR (RT-PCR) experiments were performed to check that E1, E2 and E2C transcription was not effected. The primers that were used to detect E1, E2 and E2C from cDNAs produced from mRNA extracts, did not overlap with each other or with the E1^E4 ORF (Figure 5.3a and b; Appendix V). The forward primers that were used for the detection of E2 and E2C were designed over splice junctions to avoid the amplification of unspliced E1, E2 and E4 transcripts. The reverse E2 and E2C primers did not overlap with each other or with the E4 ORF, these primers were

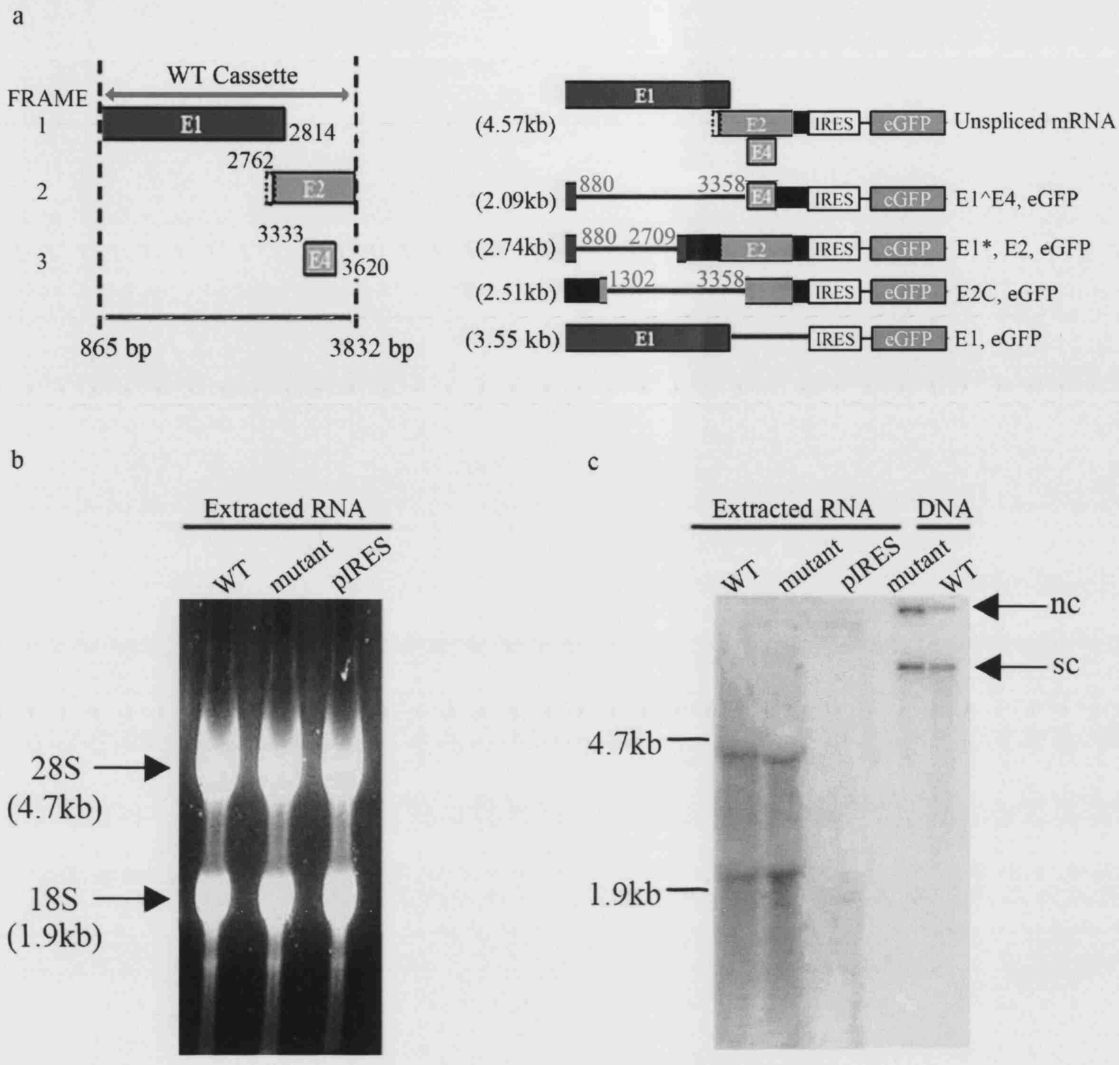


Figure 5.2 RNA transcripts produced from the WT and mutant cassettes
(a) Schematic representation of the early region of the HPV16 genome that was cloned upstream of an internal ribosome entry site (IRES) and an eGFP gene to produce the WT and mutant cassettes. The mRNAs that are thought to be transcribed from the HPV16 genome were used to predict the spliced mRNA species and their sizes (Baker and Calef, 1996). The viral and the eGFP ORFs are denoted by coloured boxes and are labelled. The positions of exons are indicated by black boxes, while the introns are indicated by thin black lines. The relative position of the IRES sequence on the RNAs is also indicated. (b) Ethidium bromide stained 1.2 % formaldehyde agarose gel of total RNA samples extracted from transfected C33a cells. The position and size of ribosomal RNAs (28S and 18S) which served as internal RNA size markers are indicated. In the last two lanes on the gel ~100 pg of pIRES.WT cassette and pIRES.mutant cassette were also electrophoresised but could not be detected. (c) Northern blot of equal quantities of total RNA extracted from transfected cells. The positions of different forms of plasmid DNA (i.e. nicked circle (nc) and supercoiled (sc)) are indicated.

designed based on the primers that were originally used to identify E2 and E2C transcripts produced from the HPV16 genome (Doorbar et al., 1990). The E1 primers used can theoretically amplify all transcripts that contain the full E1 ORF i.e. the unspliced and the putative spliced E1 transcripts (predicted to be produced but as yet have not been identified in HPV16), both the unspliced and spliced E1 messages are thought to be used for E1 protein production (Klumpp and Laimins, 1999). It was found that transcripts that encode all three of these proteins were produced by both the WT and mutant cassettes (Figure 5.3c). No PCR products were amplified from RT-PCR reactions from mRNA extracted from pIRES transfected cells or from PCRs carried out using mRNA samples directly as template (Figure 5.3d). This suggests the correct-sized PCR bands produced from the WT and mutant cassette samples were specific, and not due to amplification of pIRES or cellular cDNA or amplification of contaminating DNAs present in the mRNA sample.

5.4 Quantitation of E2/E1^E4 cytoplasmic colocalisation by immunofluorescence

Cos-7 cells were transfected with pIRES.WT cassette (WT cassette) and pIRES.mutant cassette (mutant cassette) for 18h to 72 h before being assessed for cytoplasmic E2 expression. Cos-7 cells were chosen for immunofluorescence analysis because the cytoplasm to nucleus ratio in these cells allows cytoplasmic structures to be identified easily. It was found that approximately one fifth of cells that were transfected with the mutant cassette expressed cytoplasmic E2, and that this did not change with time (Figure 5.4a). In contrast, E2 was found to accumulate in the cytoplasm of cells co-expressing E2 and E1^E4 over time (Figure 5.4b). Three E2/E1^E4 phenotypes were seen in transfected cells that were positive for both proteins; i.e. no colocalisation, partial colocalisation and total colocalisation (Figure 5.4c). In cells that showed no E2/E1^E4 colocalisation, both proteins were segregated in different compartments of the cell (Figure 5.4c). No colocalisation between E2 and E1^E4 occurred more frequently at early time points post-transfection than at later ones. Partial E2/E1^E4 colocalisation patterns accounted for the majority of colocalisation in cells transfected with the WT cassette at all the time points examined (Figure 5.4d). In these cells E2 was expressed in the nucleus and the cytoplasm, but the cytoplasmic E2 appeared to colocalise exactly in some places with E1^E4 (Figure 5.4c). The number of cells showing total cytoplasmic E2/E1^E4 colocalisation

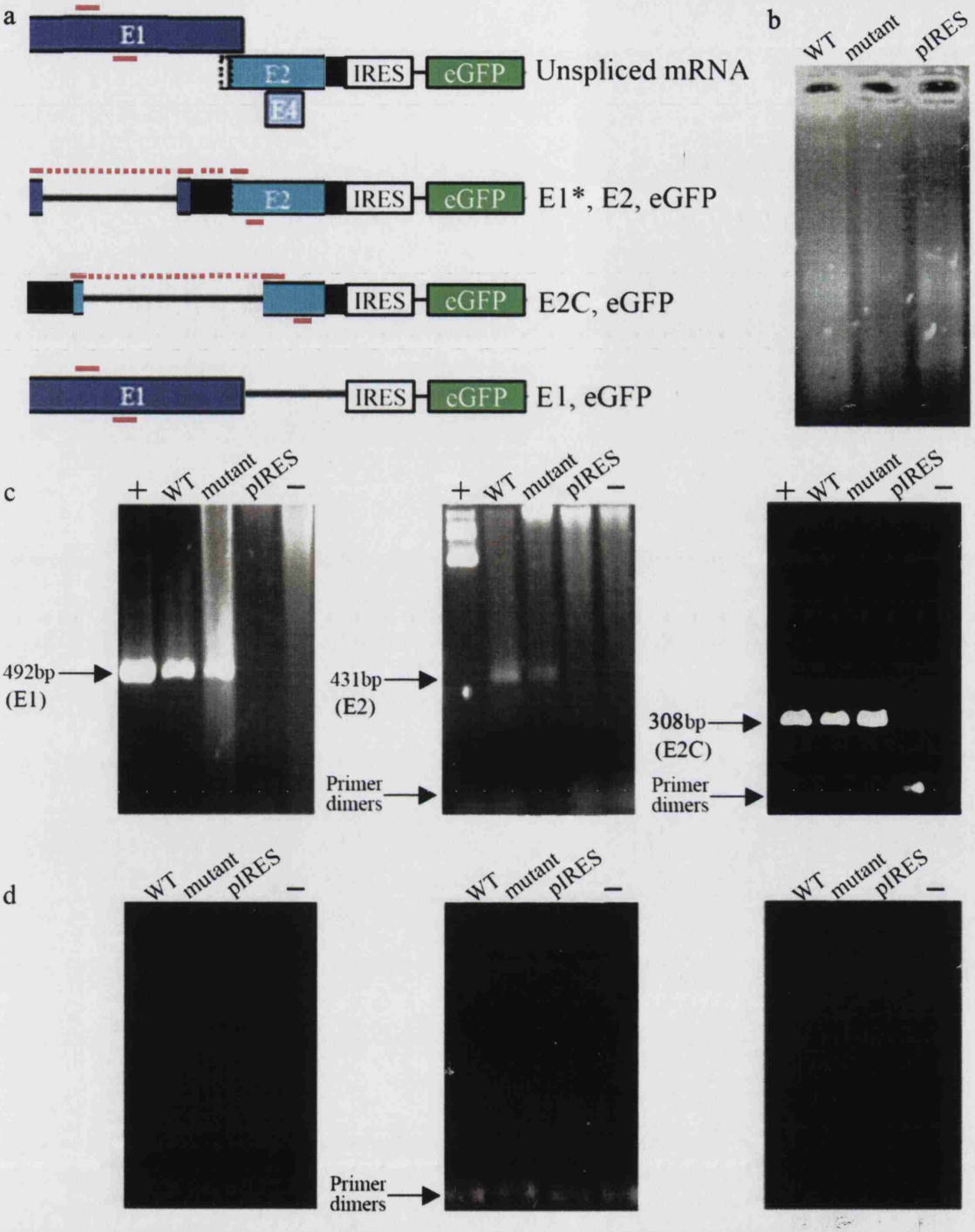
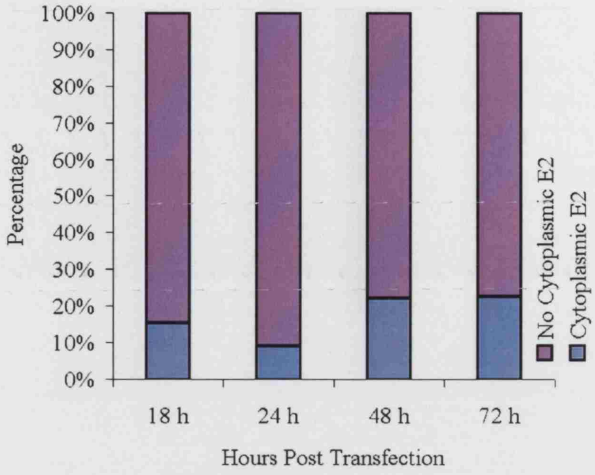


Figure 5.3 RT-PCR from mRNA produced from the WT and mutant cassettes
(a) Shown by red lines are the approximate positions of the PCR primers used to amplify E1, E2 and E2C bind on amplified cDNAs. The E2 and E2C primers were designed based on the primers that were originally used to identify E2 and E2C transcripts from HPV16 (Doorbar et al., 1990). The E1 primers can in theory amplify E1-spliced and unspliced transcripts. The exact binding positions of the primers are shown in Appendix III. (b) Ethidium bromide stained 2 % agarose gel of mRNA (smears) extracted from transfected C33 cells. (c) Ethidium bromide stained 2 % agarose gel of cDNA PCRs of the samples with E1, E2 and E2C specific primer pairs. Positive (pMV11.E1, pMV11.E2 and pcDNA.E2C) and negative (cDNA from pIRES transfected C33a cells) and no template control (-) reactions were also included. (d) Ethidium bromide stained 2 % agarose gel of mRNA PCRs of the samples with E1, E2 and E2C specific primer pairs.

Figure 5.4 Analysis of E2/E1^E4 colocalisation in the cytoplasm of cells
(a) Bar chart representing the frequency of cytoplasmic E2 at four time points post-transfection with the mutant cassette. On average 80 infected cells were counted for each time point. On the right of the graph a representative field of view of Cos-7 cells transfected for 72 h with the mutant cassette and stained for E2 with the antibody TVG261. (b) Bar chart representing the frequency of cytoplasmic E2 at four time points post-transfection with the WT cassette. On average 80 transfected cells were counted for each time point. On the right of the graph a representative field of view of Cos-7 cells transfected for 72 h with the WT cassette and stained for E2 with the antibody TVG261. (c) Representative E2/E1^E4 expression patterns produced from the WT cassette. The first row shows a cell with no E2/E1^E4 colocalisation, the second row shows a cell with partial E2/E1^E4 colocalisation and the third row shows a cell with total E2/E1^E4 colocalisation in the cytoplasm. (d) Bar chart representing the proportions of cells in part b, that are showing either partial or total E2/E1^E4 cytoplasmic colocalisation at each time point examined post-transfection.

a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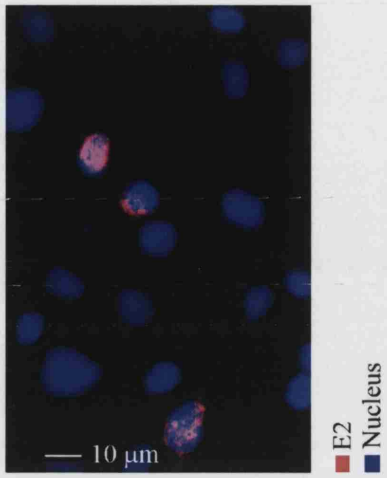
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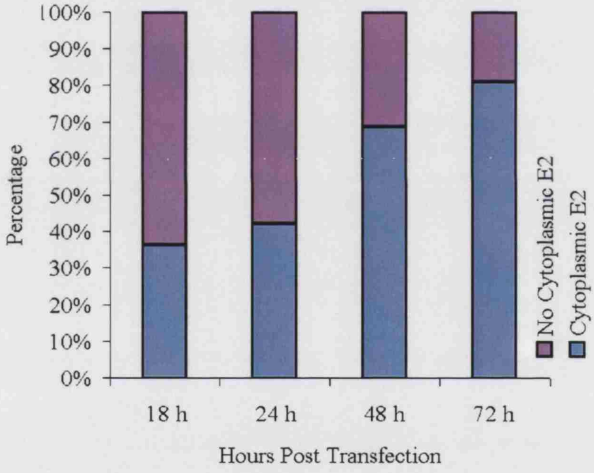
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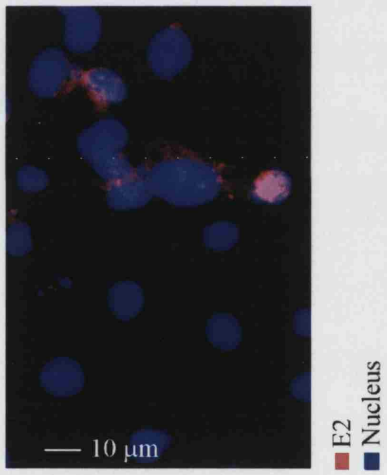
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E2
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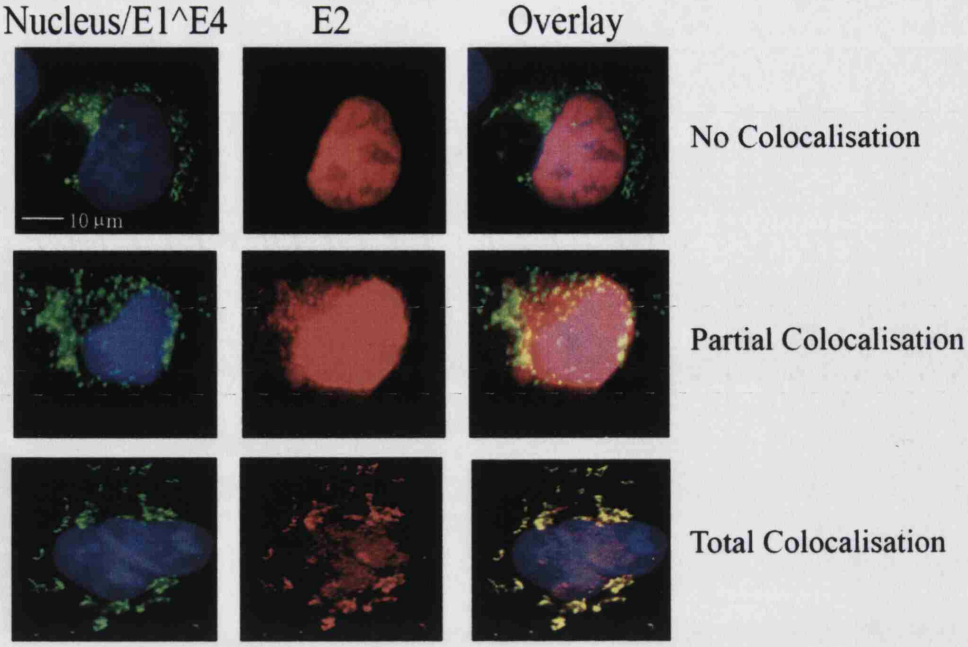
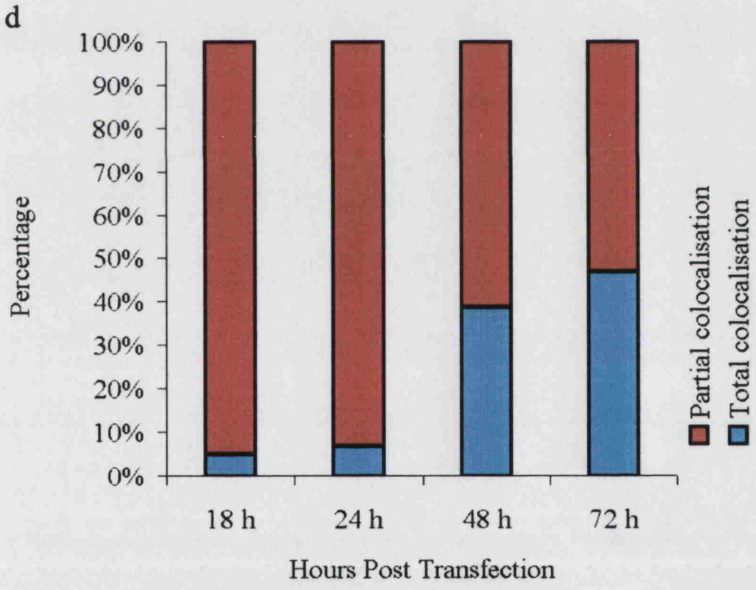

Nucleus/E1^E4
E2
Overlay
10 µm
No Colocalisation
Partial Colocalisation
Total Colocalisation

d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60%
50%
40%
30%
20%
10%
0%
18 h
24 h
48 h
72 h
Hours Post Transfection
Partial colocalisation
Total colocalisation

increased with time, accounting for almost half of the cells showing E2/E1^E4 colocalisation at 72 h post transfection (Figure 5.4d). In these cells, varying levels of diffuse nuclear E2 was observed, but in the cytoplasm, E2 and E1^E4 shared either a filamentous or more commonly, a punctate pattern (Figure 5.4c). To summarize, following transfection of cells with the WT cassette the accumulation of E2 in the cytoplasm of cells with E1^E4 structures, was observed in 42 %, 69 % and 81 % of cells at 24 h, 48 h and 72 h post transfection, respectively (Figure 5.4b). In contrast, less than 25 % of the cells transfected with the mutant cassette had any detectable cytoplasmic E2 at all the time points examined (Figure 5.4a).

5.5 Effect of the WT and mutant cassette on transcription increases with time

To test the effect of E2/E1^E4 colocalisation on transcription, luciferase assays were performed using the WT and mutant cassettes at 24 h, 48 h and 72 h post-transfection (Figure 5.5). The level of transactivation induced by the WT and mutant cassettes were similar at 24 h post transfection, but subsequently increased with time in both cases, thus transactivation appeared to increase with time of E1 and E2 co-expression, irrespective of E1^E4. However, the transactivation of the early promoter by the WT cassette was found to be ~2 fold higher than by the mutant cassette, at both 48 h and 72 h post transfection. The percentage transactivation elicited by the WT cassette at each time point was also found to correlate strongly (R^2=0.999) with the proportion of cells showing E2/E1^E4 colocalisation at each time point. This implies that the cytoplasmic sequestration of E2 by E1^E4 may positively effect transcription. To assess the importance of the levels of E1, E2, and E1^E4 proteins on transcription, different concentrations of the WT and mutant cassette DNAs were used in transcription assays.

5.6 The affect of WT and mutant cassette on transactivation is concentration-dependent

Transcription assays were performed with different concentrations of WT and mutant cassette expression plasmids. It is not thought that increasing the transfection concentration of the cassettes should change the ratio of E1 and E2 proteins produced

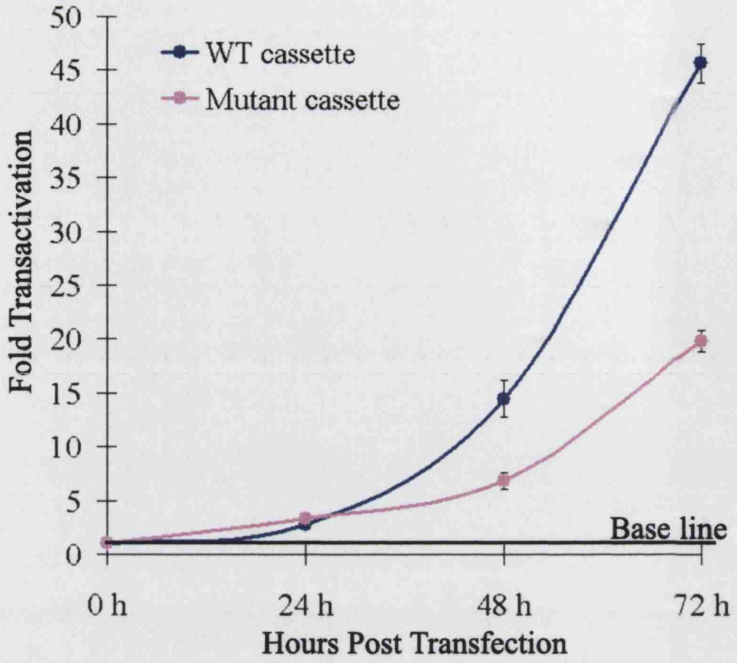


Figure 5.5 Transcription assays using WT and mutant cassettes at different time points post transfection

(a) C33a cells were transfected with 1 μg of pGL3.LCR, 1μg of phRL-TK and 7 μg of pIRES.WT cassette or pIRES.mutant cassette, for 24 h, 48 h and 72 h (NB transfections made up to 15 μg of DNA with pIRES). The samples were then assayed for luciferase activity, and total protein concentration. The graph shows the fold transactivation of each sample with respect to the basal promoter activity. This was calculated by dividing the luciferase activity for 120-130 μg of each cell extract by that for the mean value of pGL3.LCR (1 μg), phRL-TK (1μg) and pIRES (13 μg) transfected cells. The error bars shown on the graph represent the SE of three independent samples.

by either of the cassettes, but it is expected that this should be able to cause a rise in the levels of E1 and E2 protein expression.

It was found that at concentrations of ≤4 μg of WT and mutant cassettes per transcription assay, the levels of transactivation invoked by both cassettes was similar (Figure 5.6). However, at concentrations between 6 to 8 μg the level of transactivation induced by both cassettes increased linearly, and transactivation by the WT cassette increased at a rate that was approximately 4 times that of the mutant cassette. At concentrations of >8 μg of WT and mutant cassettes, the level of transactivation induced by the WT cassette was higher than by the mutant cassette, but the transactivation profiles produced by both were alike. Both WT and mutant cassette reached a transactivation plateau, but the actual level of transactivation elicited was ~2 fold higher in the WT than the mutant samples (Figure 5.6). This shows the transactivation potential of the WT cassette compared to the mutant cassette is higher.

The similarities in the transactivation profiles of both the WT and mutant cassettes suggest that the pattern of transactivation is dictated by E1 and E2 proteins. The expression of E1^E4 by the WT cassette (in addition to E1 and E2), seems to increase the level of E1/E2-mediated transactivation without changing the transactivation profile. The effect of E1^E4 on transactivation is more pronounced when concentrations of >4 μg of the cassettes were used in transcription assays. This suggests that a critical level of E1 and E2 proteins must be expressed by the WT cassette before E1^E4 can noticeably enhance E1/E2-mediated transactivation. The levels of transactivation that could be elicited by ≥6 μg of the WT cassette, could not be reached by any concentration of the mutant cassette tested, suggesting that E1^E4 expression can enhance E1/E2-mediated transactivation above the level that can be achieved by E1 and E2 alone.

E1^E4 is thought to be able to enhance HPV genome replication, and so it is possible that in these transcription assays where the viral replication proteins, E1 and E2 are present alongside pGL3.LCR which contains the HPV origin of replication (ori), that the latter may be replicated following transfection. The level of replication of the reporter plasmid mediated by the WT cassette could be greater than that achieved by

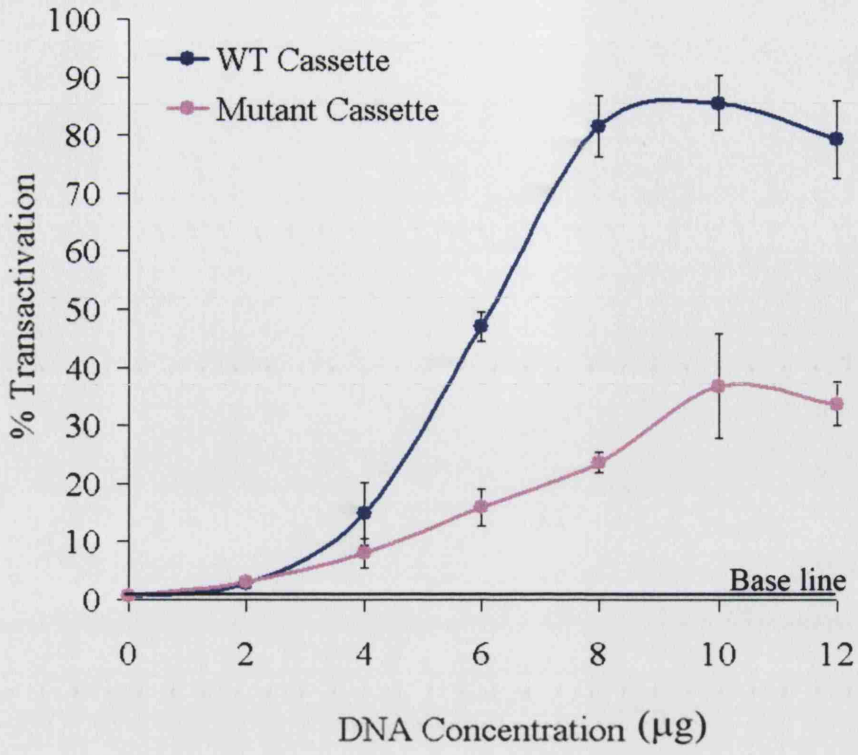


Figure 5.6 Effect of increasing WT/mutant cassette concentration on basal transcription from the LCR reporter plasmid

C33a cells were transfected with 2-12 μg of pIRES.WT cassette or pIRES.mutant cassette, 1μg of phRL-TK and 1 μg of pGL3.LCR for 72 h (NB transfections made up to 18 μg of DNA with pIRES). The samples were then assayed for luciferase activity, and total protein concentration. The graph shows the percentage transactivation of each sample with respect to the basal promoter activity. This was calculated by subtracting the mean luciferase activity for 120-130 μg of pGL3.LCR (1 μg), phRL-TK (1μg) and pIRES (17 μg) transfected cells from that of each cell extract and then converting this value to a percentage, where the highest WT cassette value in the experiment was taken as a 100 %. The error bars shown on the graph represent the SE of at least six independent samples.

the mutant cassette, due to the presence of E1^E4. This in turn could cause an increase in transactivation in the WT cassette samples due to higher levels of reporter plasmid in the WT assays compared to the mutant assays. To test whether E1^E4 actually could increase the replication of the reporter plasmid, the WT and mutant cassettes were used in replication assays.

5.7 E1^E4 enhances E1/E2-mediated replication

A role for E1^E4 in vegetative viral DNA amplification has already been suggested on the basis of its expression pattern *in vivo* (Breitburd et al., 1987; Doorbar et al., 1997). To examine the role of E1^E4 in viral replication, the ability of the proteins produced from the WT and mutant cassettes to replicate a plasmid containing the HPV origin of replication (ori) was tested. Low molecular weight (MW) DNA was extracted from transfected cells, and then digested with *Hind III* to linearise the ori-containing reporter plasmid (pGL3.LCR). Half of each sample was then digested with *Dpn I*, which selectively digests DNA at methylated GATC sites, of which there are twenty-five present in pGL3.LCR extracted from bacteria. Eukaryotic cells do not contain dam methylases which can modify the adenine base of GATC sequences, and so DNA replicated in these cells is resistant to *Dpn I* digestion (reviewed in (Marinus, 1987)).

Total pGL3.LCR and replicated pGL3.LCR isolated from transfected cells was detected by Southern blotting (Figure 5.7a and b). A *Dpn I*-resistant band could be detected in the pIRES sample as expected. However, *Dpn I*-resistant bands were apparent following co-transfection of pGL3.LCR with the mutant cassette and the WT cassette. This suggests that both the WT and mutant cassettes could replicate the ori-containing reporter plasmid in cell culture. The replicated DNA band appeared to be more intense in the WT sample compared to the mutant sample (Figure 5.7b). This suggests that E1^E4 may enhance E1/E2-mediated viral DNA replication. However, the overall level of replication that was observed in the two systems was very low and was only detectable by Southern blotting following prolonged exposure to a phosphorimager cassette. A titration of the radioactively labelled probe against different dilutions of undigested plasmid DNA was subsequently performed to estimate the levels of replicated DNA recovered following transfection. Upon comparison of the intensities of the replicated bands with the different concentrations

of plasmid DNA that could be detected by Southern blotting, it appeared as though the levels of ori-dependent replication by both the WT and mutant cassettes in C33a cells was in the range of 8 pg (Figure 5.7c). The finding that only low levels of replication were observed in C33a cells was not surprising since ori-dependent replication has previously only been reported to only occur at low levels in this type (Butz and Hoppe-Seyler, 1993; Demeret et al., 1995).

It is still possible that the slightly elevated levels of pGL3.LCR replication that were seen in WT cassette samples compared to mutant cassette samples could be responsible for the enhanced E1/E2-mediated transactivation by the WT cassette. To test this hypothesis it was important to repeat the transcription assays using WT and mutant cassettes in a population of cells that was prevented from entering S-phase, as this should allow transcription from pGL3.LCR to proceed in the absence of replication. Ori-dependent replication requires cellular factors that are only produced in S-phase of the cell cycle, thus if cells cannot enter S-phase, ori-dependent replication should be prevented.

5.8 Attempts to induce G1 arrest in C33a cells

5.8.1 Aphidicolin usage

Aphidicolin is a cell-permeable ligand that can bind to DNA polymerase α, an enzyme that is essential for the expansion and elongation of the replication bubble, in both cellular and viral DNA replication (Raff and Glover, 1988). The binding of aphidicolin to DNA polymerase α inhibits its function and causes cells to arrest at the G1/S boundary. Aphidicolin has been used successfully to prevent S-phase, without completely inhibiting transcription of a chloroamphenicol reporter gene under the control of the HPV18 LCR, in similar assays (Demeret et al., 1998). For this reason it was decided to use aphidicolin to inhibit the replication of pGL3.LCR in transcription assays performed with the WT and mutant cassettes. In these experiments it was important to arrest the cells in G1, before high levels of E1^E4 protein, were expressed in the cells. This is because it has previously been shown that cells expressing high levels of 16E1^E4 protein can cause cells to arrest in G2 (Davy et al., 2002).

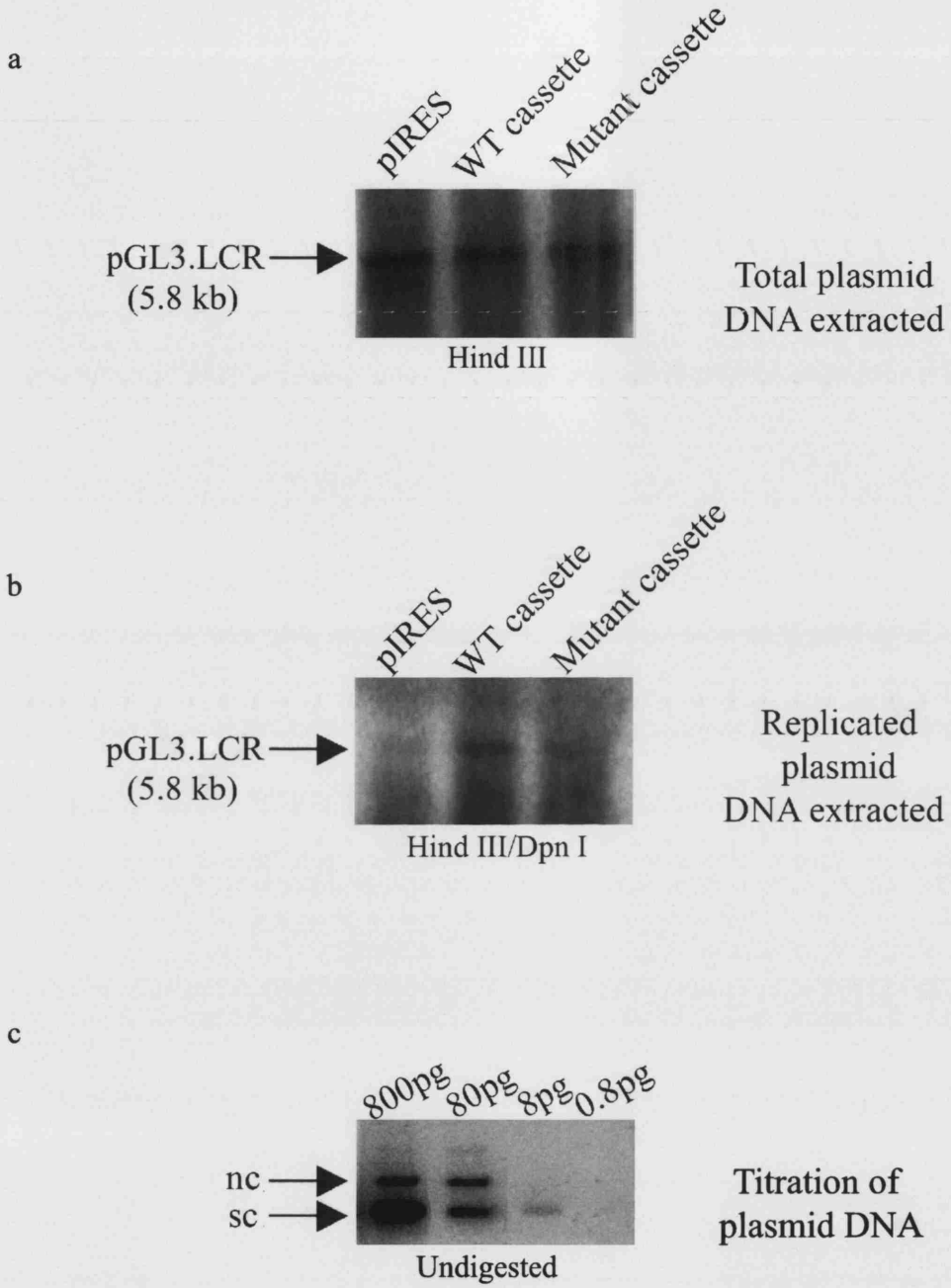


Figure 5.7 Effect of E1^E4 on the E1/E2-mediated replication of pGL3.LCR
C33a cells transfected with 1μg pGL3.LCR, 1μg of phRL-TK and 7 μg of pIRES.WT cassette or pIRES.mutant cassette or pIRES (NB transfections made up to 13 μg of DNA with pIRES) for 72 h. (a) Southern blot of total low MW DNA extracted from transfected cells and linearised by *Hind III* digestion. (b) Southern blot of replicated low MW DNA extracted from transfected cells digested with *Hind III* and *Dpn I*. (c) Southern blot of different dilutions of undigested plasmid DNA.

Prior to transfection, C33a cells were maintained as sub-confluent populations in which cells could be found in all stages of the cell cycle (Figure 5.8a). The mitotic cells in a population are the most likely to internalise and transport precipitated DNA to the nucleus following transfection (Pellegrin et al., 2002). So it was important to transfect cells prior to treatment with aphidicolin. Transfection reagents are also renowned for affecting the cell cycle, so the effect of calcium phosphate precipitates on C33a cells was analysed using flow cytometry (Renzing and Lane, 1995; Rodriguez and Flemington, 1999; Jung and Flemington, 2001). It was found that transfection of C33a cells caused some cell death, as indicated by an accumulation of cells with sub-G1 DNA content (i.e. fragmented DNA, characteristic of dead and dying cells), but no other significant effect was seen compared to untransfected cells at the same time-point (Figure 5.8b and c).

Aphidicolin (2 μg ml^{-1}) was added to the media 36 h post-transfection and left on the cells for the remainder of the experiment (36 h). Aphidicolin was added at this time point to allow G1/S arrest to occur before high levels of E1^E4 and E2 were expressed which could arrest the cell cycle in G2 (Fournier et al., 1999; Davy et al., 2002). However, it was found that the prolonged incubation of cells with aphidicolin following transfection, resulted in the majority of adhered cells having sub-G1 DNA contents, which suggested the conditions used in the experiment were beyond the tolerance of the cells (Figure 5.8d).

Aphidicolin-related apoptosis has been reported previously following prolonged exposure of cells to the drug (Kung et al., 1990). Although it seemed possible that the parameters used for studying the effects of E1^E4 on E1/E2-mediated transactivation and aphidicolin use could be optimised, the difficulties that were encountered prompted us to use another method to examine p97 activity in the absence of ori-dependent replication.

5.8.2 Serum starvation

Signals external to the cell can promote cell cycle progression. These signals can be provided to cultured cells by growth factors present in serum-supplemented media

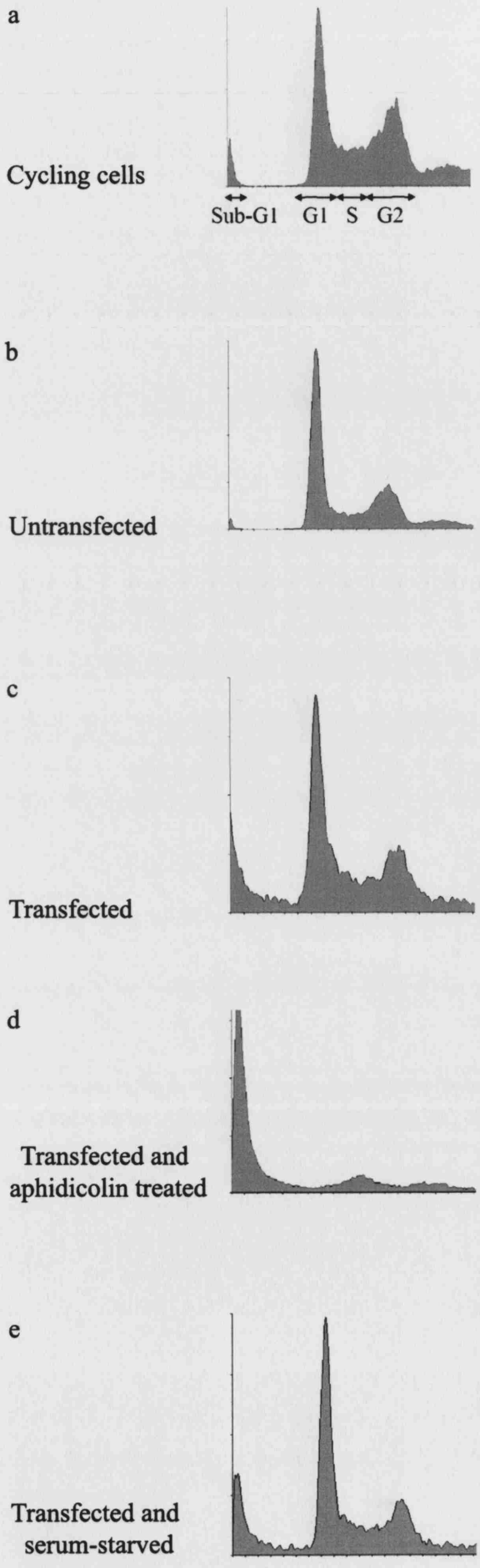


Figure 5.8 Attempts to G1 arrest transfected C33a cells

C33a cells were seeded at a density of 2.5 x 10^5 cells per a well of each 6-well dish, and were harvested 96 h afterwards unless specified. Cells were stained with propidium iodide and analyzed by flow cytometry (This was performed Dr. Deb Jackson, NIMR). Histograms showing the number of cells (on the y axis) having a particular size (as measured by forward scatter) and particular DNA content (as measured by red fluorescence), were obtained. (a) Untransfected cells harvested 24 h post seeding. The position and proportions of the cell populations in different cell cycle phases has also been indicated below the profile. Cells with less than 2N DNA content are labelled sub-G1, and the cells labelled as G1 could also be in G0. (b) Untransfected cells (c) Transfected cells (pIRES) for 72 h. (d) Transfected cells (pIRES) for 72 h, treated with aphidicolin for 36 h. (e) Transfected cells (pIRES) for 72 h, serum starved cells for 36 h.

(Hung et al., 1996). In a previous study, C33a cells were deprived of serum for a period of 36 h and the authors claimed that this caused the cells to arrest in the G1 phase of the cell cycle (Truscott et al., 2003). This mode of inducing cell cycle arrest seemed theoretically to be compatible with the established transcription assay method, and so serum starvation was combined with transfection and the effect on the cell cycle was assessed (Figure 5.8e). However, in our experiments no significant differences were observed between the proportions of cells in the G0/G1 phases of the cell cycle following serum starvation compared to those that were not deprived of serum (Figure 5.8c and e). This suggests that depriving the cells of serum did not cause G1 arrest in our experiments.

It was proving to be a difficult task to separate the possible effects of E1^E4 on E1/E2-mediated transcription from the effects of E1^E4 on replication of the ori-containing reporter plasmid. To circumvent this problem it was decided to test the effect of the WT and mutant cassettes on transactivation from an artificial E2-responsive promoter, which lacked the E1 binding site and thus would be replication defective in eukaryotic cells. The reporter plasmid that was chosen contained six consensus E2 binding sites ($ACCG(A)_4CGGT$) upstream of a thymidine kinase promoter of Herpes Simplex Virus (HSV) (Figure 5.9a, a kind gift from Roland Sahli, Institute of Microbiology, Lausanne, Switzerland; (construct is described in Appendix IV). The lack of an E1 BS also means that E1 cannot bind to the regulatory region of the promoter directly, but E1 may still be able to act as a co-transcription factor via E2 binding.

5.9 The effect of E1^E4 on E2-mediated transactivation in the absence of replication

Transcription assays using the WT and mutant cassettes transfected with pTK-luc were performed in C33a cells, to allow the level of transactivation induced by the WT and mutant cassettes to be compared in the absence of replication (Figure 5.9b). Using this system it was found that the WT cassette could elicit approximately a 3-fold higher level of transactivation than the mutant cassette in analogous experiments. This result was found to be initially surprising, as the activity of the artificial promoter

was expected to increase with higher nuclear E2 concentrations, as none of the E2 BSs had been shown to be repressive (Thierry et al., 1990). Thus, it was expected that ability of the WT cassette to cause the cytoplasmic accumulation of E2 and in turn reduce nuclear E2 levels would decrease the level of transactivation by the WT cassette compared to the mutant cassette (Figure 5.4c).

The reduction in nuclear E2 has been shown qualitatively in these experiments, but several fractionation protocols were attempted to try and quantitatively assess the levels of nuclear E2 in transfected cells (data not shown). However, these experiments were unsuccessful (i.e. cellular cytoplasmic or nuclear markers were repeatedly found in the wrong cell fractions). The results of these experiments reflect the innate difficulties that have also been encountered by other researchers, in the fractionation of 16E1^E4 expressing cells (Davy et al., 2005).

Transactivation assays using pTK-luc and the WT and mutant cassettes showed that E1^E4 expression could increase E2-mediated transactivation, independently of any effect of E1^E4 on replication. It was unclear though from this experiment how E1^E4 could increase the activity of the non-repressive artificial E2-responsive promoter in these assays by reducing nuclear E2 levels. It is conceivable that the ability of E1^E4 to adjust the nuclear levels of E2 may enhance transactivation by optimising the nuclear ratio of E2 to its co-transcription factor E1 in this system. This would allow the WT cassette to enhance transcription above the level elicited by the mutant cassette. Although it is possible a difference in E2 or maybe even E1 protein levels between the cassettes could also effect transactivation.

5.10 Recombinant protein expression by the WT and mutant cassette expression vectors

Total cell protein extracted from C33a cells transfected with the empty expression vector (pIRES), or the WT or mutant cassettes was analysed by Western blotting (Figure 5.10). Antibodies that recognised 16E1, 16E2, 16E1^E4 and eGFP were used to detect the range of full length proteins encoded by the WT and mutant cassettes. Tubulin levels were also monitored in each sample as a loading control. The E1 protein was not detected in either the WT or mutant cassette samples, but was in the

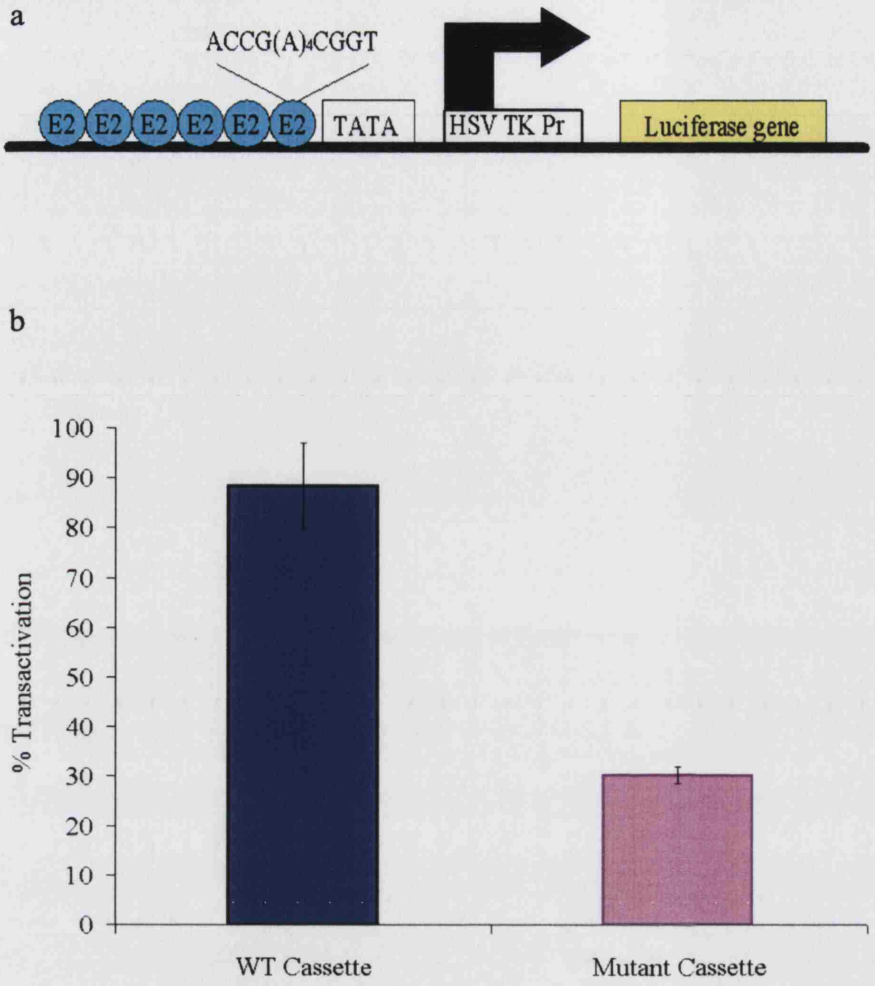


Figure 5.9 Transactivation of an E2-responsive artificial promoter by the WT and mutant cassettes

(a) Schematic of the E2-responsive reporter plasmid (pTK), downstream of the herpes simplex virus thymadine kinase (TK) promoter (Appendix IV). The promoter used contains 109 nucleotides upstream of the start site of the TK gene. Upstream of the promoter (position -32) six identical E2 BSs (sequence shown) were cloned in tandem. (b) C33a cells were transfected with 1μg of phRL-TK and 1 μg of pTK-luc 7 μg of pIRES.WT cassette or pIRES.mutant cassette for 72 h (NB transfections made up to 15 μg of DNA with pIRES). The samples were then assayed for luciferase activity, and protein concentrations. The graph shows the percentage transactivation of each sample with respect to the basal promoter activity. This was calculated by subtracting the luciferase activity for 120-130 μg of pTK (1 μg), phRL-TK (1 μg) and pIRES (11 μg) transfected cells from that of each cell extract and then converting this value to a percentage, where the highest value in each experiment was taken as a 100 %. The error bars shown on the graph represent the SE of at least six independent samples.

positive control (Figure 5.10a). This is not surprising as E1 is an elusive protein that is only expressed at low levels both *in vitro* and *in vivo* due to the presence of a strong splice donor site at the beginning of the E1 ORF (Grassmann et al., 1996; Remm et al., 1999; Wang et al., 2003). The E1^E4 protein was detected in the WT sample but not in the mutant sample, and eGFP was expressed at equivalent levels by both the WT and mutant cassettes (Figure 5.1c and 5.10). In contrast, the full length E2 and the E2 N-terminus fragment appeared to be reproducibly present at higher levels in the WT sample (~2 fold as assessed by densitometry with respect to the transfection controls) compared to the mutant sample despite equal sample loading (Figure 5.10). This difference in E2 levels between the cassettes could possibly provide an explanation for why the transactivation ability of the WT cassette was higher than that of the mutant cassette in comparative assays. This difference that was observed in E2 protein levels could be due to either a difference in E2 mRNA transcription or E2 protein stability between the two cassettes. To assess this, the relative levels of E2 transcripts in the WT and mutant cassette samples needed to be quantified. For completeness the relative levels of E1 transcripts in the WT and mutant cassette samples also needed to be assessed.

5.11 E1 and E2 transcription by the WT and mutant cassettes

Messenger RNA (mRNA) extracted from cells transfected with either pIRES, the WT cassette or the mutant cassette was used as template for quantitative reverse-transcriptase polymerase chain reactions (qRT-PCR). E1 and E2 transcripts were detected in both WT and mutant samples but not in the non-template control (NTC) reactions or in the negative control samples (pIRES transfected and untransfected). Figure 5.11a and 5.12a show the amplification plot for the WT and mutant cassette qRT-PCRs and representative control reactions. To confirm that the products that were amplified using E1 and E2 specific primers were specific, a representative qRT-PCR WT cassette product from each experiment was examined by agarose gel electrophoresis. It was found that in both qRT-PCRs only one product of the expected size was amplified, this suggested that the E1 and E2 primers that were used in these experiments were specific (Figure 5.11b and 5.12b; Appendix V). The relative mean abundance of E1 and E2 transcripts present in all the samples tested are shown in

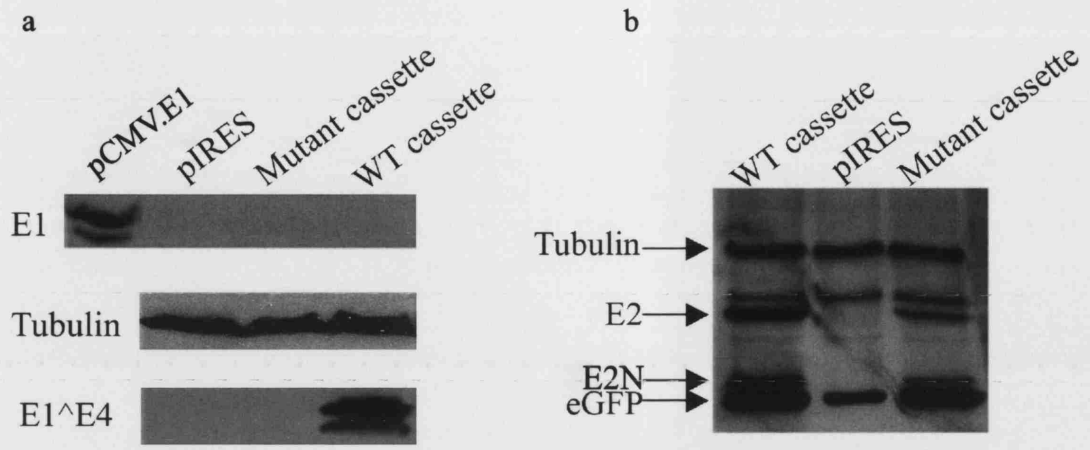


Figure 5.10 Western blots of proteins produced from the WT and mutant cassettes

Western blots of ~25 μg of the SDS fraction of C33a cells transfected with 7 μg pIRES, pIRES.WT cassette or pIRES.mutant cassette for 72 h as labelled (NB transfections made up to 15 μg of DNA with pIRES). (a) Top panel is an E1 Western blot using the antibody E1-N1. The positive control on the blot was C33a cells transfected with a codon-optimised 16E1 expression vector, pCMV.E1 (A kind gift from Dr. McClements, Department of Microbial Vaccine Research, West Point, USA). The bottom panel is an E1^E4 Western blot using the antibody TVG402. The middle panel is a tubulin Western blot using an anti-tubulin monoclonal antibody, this served as a loading control for the E1 and E1^E4 Western blots. (b) Western blot of E2 using the antibody TVG261, eGFP and tubulin with specific monoclonal antibodies (Section 2.6.2.11). Tubulin served as a loading control for the samples and eGFP as a transfection control.

Figure 5.11c and 5.12c, respectively. In both cases a slight reduction in the mean level of both E1 and E2 transcripts were seen in the mutant cassette sample compared to the WT sample, however these differences were not found to be statistically significant in t-test with a confidence interval of 99 % (Appendix VI). This suggests that the differences observed in transactivation and E2 protein levels between the cassettes are unlikely to be a result of differences in the levels of E1 and E2 encoding transcripts.

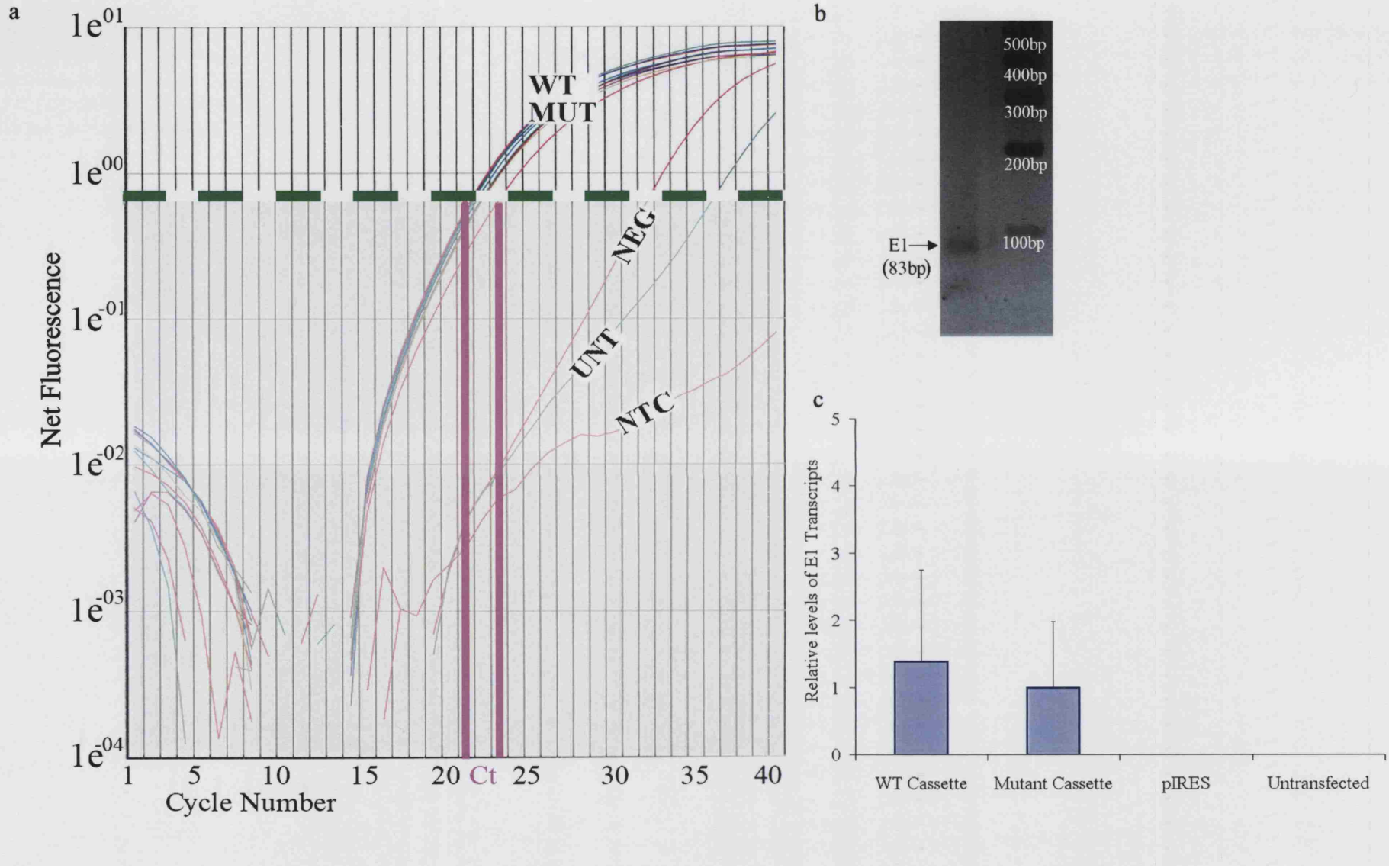
5.12 Discussion

The early cassette system was used to express E1, E2 and E1^E4 to allow all three of the proteins to be produced in cells at a ratio that resembled that found *in vivo*. When E1^E4 was expressed with E1 and E2 at high levels from the WT cassette, it was found that E1^E4 could cause an increase in E1/E2-mediated transactivation. However, at low protein concentrations the expression of E1^E4 with E1 and E2 by the WT cassette did not increase the level of transcription from the early promoter compared to the mutant cassette. Our results suggest that when E1, E2 and E1^E4 proteins are expressed together at high levels, E1^E4 may play a role in the regulation of the early promoter. Thus, the modulation of p97 activity by E1, E2 and E1^E4 is more likely to be important in the late stages of the virus life cycle when all three of these proteins are expressed together at high levels in the same cells.

The E2 protein was produced by both the WT and mutant cassettes, but E2 was only found to accumulate in the cytoplasm of cells with time, in cells expressing the WT cassette. In these cells E2 accumulated at E1^E4 structures in the cytoplasm to varying extents, but the complete sequestration of E2 out of the nucleus was not observed. Coincident with an increase in the frequency of E2 and E1^E4 colocalisation, a parallel increase in the level of E1/E2-mediated transactivation was also observed. This suggested that the cytoplasmic sequestration of E2 by E1^E4 may be linked somehow to the increase seen in transactivation in E2 and E1^E4 expressing cells. The data obtained in the previous chapter suggested that the association between E2 and E1^E4 in the cytoplasm could modulate E1/E2-mediated transactivation by adjusting the nuclear ratio of E2 to E1. This hypothesis is supported by the findings in this chapter. The observation that E2 protein levels were

Figure 5.11 Quantitative RT-PCR (qRT-PCR) of E1 mRNA produced by WT and mutant cassettes

C33a cells were transfected with empty vector (pIRES), pIRES.WT cassette or pIRES.mutant cassette for 72 h. The mRNA was extracted from these samples and also from an untransfected cell population and was used to produce cDNA templates in qRT-PCR reactions. (a) Amplification plot of 5 replica WT and mutant (MUT) RT-PCR samples and one representative untransfected (UNT) and non-template control (NTC). The broken green line marks the threshold value taken for the PCR reaction. The cycle number at which the net fluorescence for each sample crosses the threshold line is called the threshold cycle (Ct). The Ct values for the mutant and WT samples are flanked by pink lines on the plot. (b) Ethidium bromide stained 2 % agarose gel of a WT cassette cDNA PCR with E1 specific qRT-PCR primer pairs. (c) Graph showing the relative difference in E1 transcripts produced from the WT and mutant cassettes. The error bars represent the SE of 5 replica samples.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300bp
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(83bp)
c
Relative levels of E1 Transcripts
0
1
2
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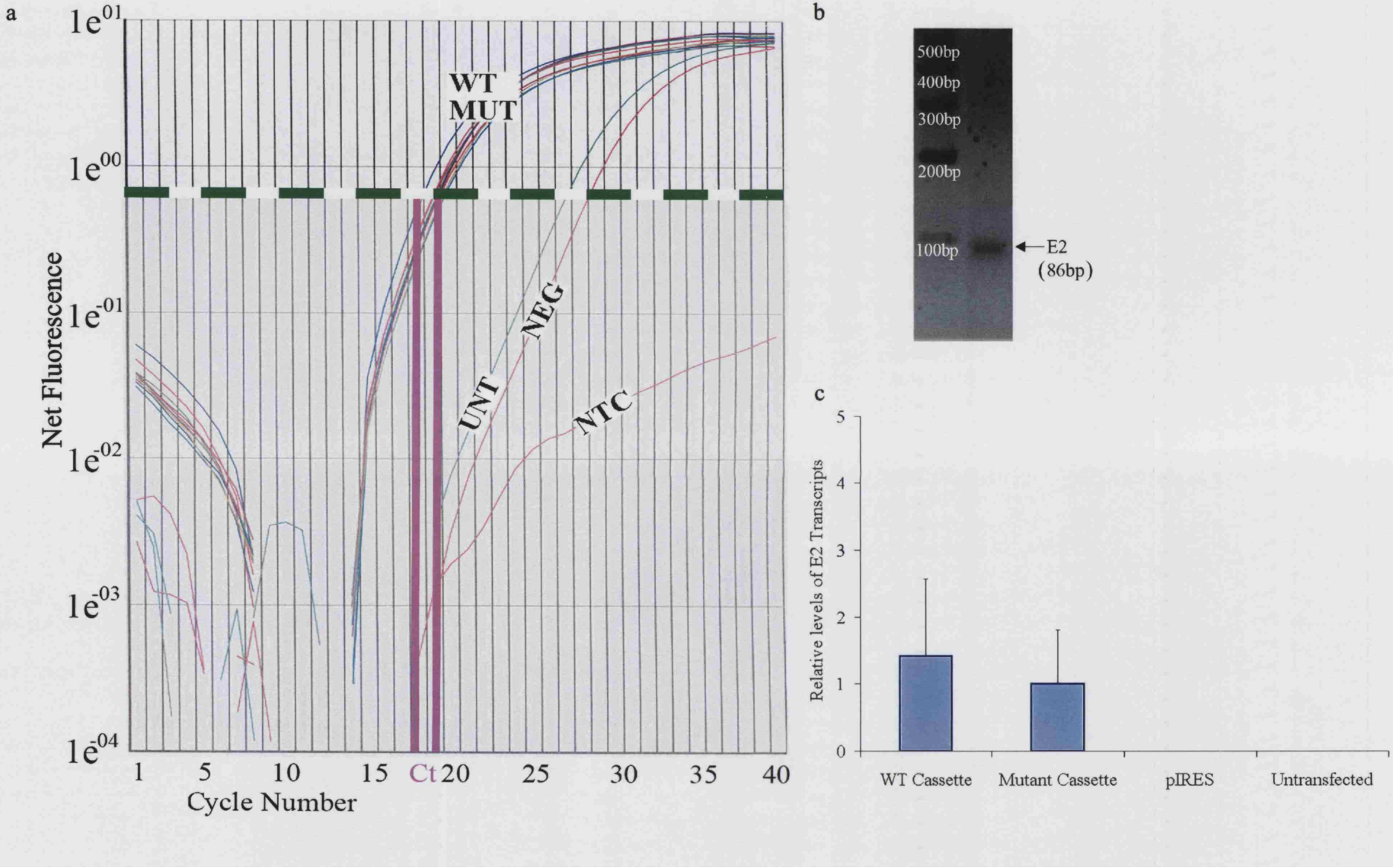
4
5
WT Cassette
Mutant Cassette
pIRES
Untransfected

Figure 5.12 Quantitative RT-PCR (qRT-PCR) of E2 mRNA produced by WT and mutant cassettes

C33a cells were transfected with empty vector (pIRES), pIRES.WT cassette or pIRES.mutant cassette for 72 h. The mRNA was extracted from these samples and also from an untransfected cell population and was used to produce cDNA templates in qRT-PCR reactions. (a) Amplification plot of 5 replica WT and mutant (MUT) RT-PCR samples and one representative untransfected (UNT) and non-template control (NTC). The broken green line marks the threshold value taken for the PCR reaction. The cycle number at which the net fluorescence for each sample crosses the threshold line is called the threshold cycle (Ct). The Ct values for the mutant and WT samples are flanked by pink lines on the plot. (b) Ethidium bromide stained 2 % agarose gel of a WT cassette cDNA PCR with E2 specific qRT-PCR primer pairs. (c) Graph showing the relative difference in E2 transcripts produced from the WT and mutant cassettes. The error bars represent the SE of 5 replica samples.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pIRES
Untransfected

found to be reproducibly higher in cells transfected with the WT cassette compared to the mutant cassette, despite no significant difference being found in E2 transcripts, suggest that the co-expression of E2 and E1^E4 may be able to stabilise E2 protein. E2 turn-over is normally tightly regulated by the ubiquitin-proteasome pathway, which has been shown to result in the rapid loss of accumulated E2 in transfected cells (Penrose and McBride, 2000; Bellanger et al., 2001). We propose that the sequestration of E2 in the cytoplasm to E1^E4 bound structures may reduce nuclear E2 levels, by decreasing the solubility of E2. The tethering of E2 to E1^E4 in the cytoplasm may also increase the stability of the E2 protein, and thus may serve as a mechanism of preventing the loss of E2, following high levels of E2 protein expression.

The WT cassette was found to cause a reproducible increase in transactivation from the p97 promoter above the mutant cassette (when transfected at ≥4 μg). The cytoplasmic sequestration of E2 by E1^E4 in these cells could act to optimise the nuclear ratio of E2 to E1 in cells transfected with the WT cassette as proposed in the previous chapter, but in addition, it is possible that the cytoplasmic E2 bound to E1^E4 in these cells may also be stabilised. If this is the case this could result in a larger number of transfected cells expressing E2 from the WT cassette compared to the mutant cassette at 72 h post-transfection. However, further experimentation is needed to be sure of this.

Another possibility that needs to be investigated further is the possibility that in addition to binding to the full length E2 protein, E1^E4 may bind and cause the relocation of E2 splice variants such as E2C in the cytoplasm. In light of *in vitro* pull-down data provided by Helena Sterlinko Grm, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy, which shows that the C-terminal domain of E2 is thought to be involved in the interaction with E1^E4. The effect of E2C on transcription may be important in the transcription assays that were performed using the WT and mutant cassettes because both cassettes were shown to produce E2C. E2C lacks the N-terminal domain of the protein and has been shown experimentally to inhibit transcription (Doorbar et al., 1990; Bouvard et al., 1994). This is thought to be due to the ability of E2C homodimers and heterodimers (formed

with the full length E2 protein), which lack the N-terminal transactivation domain (TAD) of E2 to compete with E2 homodimers in the cell for DNA binding.

The effect of E2C on E1/E2-mediated transcription has not been addressed directly in this study. However, it is conceivable that an E2/E2C heterodimer could reduce the efficiency of E1/E2-mediated transactivation. Transcripts encoding E2C were found to be produced by both the WT and mutant cassettes in this study, and previously by the HPV16 W12e genome (Doorbar et al., 1990). Thus it is possible that E1^E4 could also cause the relocation of E2C protein to the cytoplasm, and in this way could enhance E1/E2-mediated transcription. The caveat to this hypothesis is that although 16E2C transcripts have been shown to be produced from the HPV16 genome, 16E2C protein has not yet been identified *in vivo* or following transfection by the cassette. However, in support of this hypothesis an E2C truncated protein has been shown to be produced from BPV1 (Hubbert et al., 1988; Choe et al., 1989)

E1^E4 was also found to increase the replication of the reporter plasmid used in transcription assays, which contained the origin of HPV replication (ori), it is possible that the G2 arrest function of E1^E4 could contribute to the enhanced replication that was observed in E1^E4 expressing cells (Davy et al., 2002). However, it is also a possibility that the E2/E1^E4 interaction may directly be able contribute to ori-dependent replication as well. The function of E2 in replication is to target E1 to the ori and to initiate the formation of an active E1 helicase by bending the DNA at the ori in a way that encourages E1 oligomerisation (Sverdrup and Khan, 1994; Sverdrup and Khan, 1995; Gillitzer et al., 2000; Chen and Stenlund, 2001). Following this, it is imperative for the progression of replication that E2 is lost to enable E1 to be assembled into an active complex (Lusky et al., 1994; Titolo et al., 1999). This situation is analogous to the loading of the cellular replication helicase (MCMs) onto DNA by cdc6. Post loading cdc6 is lost from the nucleus following its phosphorylation by cyclin A/cdk 2 (Petersen et al., 1999; Yim et al., 2003). Similarly, the sequestration of E2 in the cytoplasm by E1^E4 could possibly serve as a mechanism to reduce E2-mediated inhibition of ori-dependent replication and in this way directly facilitate replication. The ability of the reporter plasmid to replicate in the presence of both the WT and mutant cassettes albeit at low levels could affect the

levels of transactivation that were observed, especially given that the WT cassette caused a modest increase in replication over that achieved by the mutant cassette. Despite numerous attempts we were unable to separate the effect of differential replication of the LCR-containing reporter plasmid in the WT and mutant cassette samples from replication. Instead, an artificial E2-responsive promoter was utilised, which unlike the LCR regulated reporter, could not be repressed. It was found that the WT cassette could increase transactivation of the E2-responsive promoter by approximately three times that what was achieved by the mutant cassette. This suggests that E1^E4 can enhance E2-mediated transactivation independently of any effect it may have on replication.

6 Discussion

6.1 Summary of results

6.1.1 E1^E4 binding proteins

Three different forms of HPV16 E1^E4 (16E1^E4) are detected following transient E1^E4 expression in cell culture by SDS-PAGE and Western blotting. These were the full-length or unphosphorylated form of E1^E4 and two forms of E1^E4 which were post-translationally modified by phosphorylation or cleavage. The different forms of E1^E4 varied in their solubilities and migration following SDS-PAGE, in that the unphosphorylated form of E1^E4 (10K) appeared to be more soluble than the phosphorylated (13K) and the N-terminally cleaved (8K) forms of E1^E4 (Wang et al., 2004).

An antibody which recognised all these different forms of E1^E4 was used to immunoprecipate 16E1^E4 following its expression in HPV16-transformed epithelial cells (SiHa). This resulted in the specific co-immunoprecipation of nine proteins with E1^E4. Based on the approximate sizes of these proteins, it was possible that the ~69 K protein identified was the known E1^E4 binding partner, E4-DBP, and that the ~15 K protein was possibly the HPV16 E7 protein (given that E7 was also shown to bind to E1^E4 in this study by a direct interaction, and that E7 is present in SiHa cells). It was interesting to find that the soluble and insoluble forms of E1^E4 appeared to co-immunoprecipate different repertoires of cellular proteins, suggesting that the different forms of E1^E4 may associate with different proteins in the cell.

E1^E4 was also shown to bind directly to several HPV16 proteins in this study, namely E2, E7, L1 and L2. These proteins and E1^E4 itself, displayed different binding specificities for the differentially phosphorylated forms of E1^E4. It was found that E2, L1 and L2 were all able to bind to both the phosphorylated and unphosphorylated forms of E1^E4. Of these proteins E2 and L1 appeared to associate equally well with both forms of E1^E4, whereas L2 bound preferentially to the unphosphorylated form. In contrast, E1^E4 itself and E7, were only found to associate with the unphosphorylated form of the E1^E4 protein. The interaction between E2 and E1^E4 was characterised further in this study, revealing that the binding of E1^E4 to E2 could be perturbed by the deletion of amino acids 84-88 or

86-92 of E1^E4. In addition, it was shown that the deletion of residues in the central portion of E1^E4 (amino acids 31-68), could also reduce the robustness of the interaction between E1^E4 and E2, and in particular the deletion of amino acids 36-41 considerably reduced the ability of E1^E4 to bind to E2. The capacity of the E2 and E1^E4 proteins of HPV18 to interact was also tested. Although HPV18 is evolutionary divergent from HPV16, the interaction was found to be conserved between the E2 and E1^E4 proteins encoded by this HPV type. HPV18 E2 was also shown to associate with HPV16 E1^E4, suggesting that the interaction between E2 and E1^E4 may involve conserved amino acid residues in both proteins.

6.1.2 The co-expression patterns of E2 and E1^E4 proteins in cell culture

E1^E4 is predominantly a cytoplasmic protein that colocalises with keratins and mitochondria, and E2 is a predominantly nuclear protein but it is sometimes detected in the nucleus and in the cytoplasm of cells. The co-expression of E2 and E1^E4 resulted in three phenotypes, i.e. no colocalisation, partial colocalisation and total colocalisation between the two proteins in the cytoplasm. In cells that showed no E2/E1^E4 colocalisation in the cytoplasm, both proteins were segregated in different compartments of the cell. Partial cytoplasmic E2/E1^E4 colocalisation patterns accounted for the majority of colocalisation in E2/E1^E4 expressing cells. In these cells E2 was expressed in the nucleus and the cytoplasm, but the cytoplasmic E2 appeared to colocalise exactly in some places with E1^E4. In cells that showed total cytoplasmic E2/E1^E4 colocalisation, varying levels of diffuse nuclear E2 were observed, but in the cytoplasm, E2 and E1^E4 overlapped exactly, and commonly shared a punctate pattern.

The frequency of the cytoplasmic accumulation of E2 to E1^E4 structures increased in a time-dependent manner i.e. no colocalisation between E2/E1^E4 was more common at 24 h than at 48 h or 72 h of E2/E1^E4 co-expression. However, even at late time points, the accumulation of cytoplasmic E2 in E1^E4 expressing cells rarely resulted in the complete loss of nuclear E2. Instead only a qualitative difference was observed in nuclear E2 levels (as determined by immunofluorescence) in E2/E1^E4 expressing cells over time. In this study it was also shown that the ability of E1^E4 to cause the cytoplasmic accumulation of E2 was not inhibited by the co-expression of the 16E1 protein.

6.1.3 The effect of E1^E4 on replication

E1^E4 expression with E1 and E2, resulted in a modest increase in the level of ori- (HPV origin of replication) dependent replication compared to when E1 and E2 proteins were expressed in the absence of E1^E4 for 72 h.

6.1.4 The effect of E1^E4 on transcription

E1^E4 was found to have pleiotropic effects on the activity of the HPV16 early promoter (p97), depending on whether it was expressed alone or in combination with E1 and E2. E1^E4 was found to repress p97 activity when it was expressed alone or with either E1 or E2. However, when E1^E4 was expressed with both E1 and E2 at ratios controlled by transcription using the wild-type (WT) cassette, E1^E4 was found to cause an increase in p97 activity compared to when E1 and E2 proteins were expressed in the absence of E1^E4 at late time points (48 h and 72 h) post transfection but not at an earlier time point (24 h). Thus the time for which E1 and E2 proteins were co-expressed with E1^E4 also appeared to influence the effect of E1^E4 on transactivation.

The level of E1/E2-mediated transactivation increased with time irrespectively of E1^E4 expression. However, in cells that were expressing E1, E2 and E1^E4 using the WT cassette, the rate of increase was double that found in cells expressing E1 and E2 in the absence of E1^E4 i.e. using the mutant cassette. The magnitude of the positive effect of E1^E4 on p97 activity was also found to vary with the levels of E1, E2 and E1^E4 expression, despite no change in the ratios of protein expression. The increase in transactivation by the WT cassette varied between two and four times that achieved by mutant cassette, when transfected using the same conditions. Thus E1^E4 was able to increase the potential of E1/E2-mediated transactivation above that which could be achieved by E1 and E2 alone.

It was also shown that when the WT and mutant cassettes were transfected at a constant level, the WT cassette caused the same fold increase in transactivation over the mutant cassette at both 48 h and 72 h post-transfection. Thus, although the overall level of transactivation using both cassettes was the highest at 72 h, the fold difference in transactivation between the two cassettes was similar at 48 h and 72 h post-

transfection. The contributory effect of differences in replication between the WT and mutant cassettes on transactivation was not determined. However, it was shown that E1^E4 expression from the WT cassette could have a positive effect on E1/E2-mediated transactivation following 72 h of recombinant protein co-expression, which was completely independent of replication.

The effect of methodically changing the expression ratios of E1, E2 and E1^E4 on p97 activity was also addressed. E1 caused an increase in E2 mediated transactivation at all the concentrations of E1 and E2 tested, but the level of E1/E2-mediated transactivation varied with E2 concentration. However, when E1^E4 was expressed with a fixed concentration of E1 and low concentrations of E2, E1^E4 had no effect on transactivation. This suggests that E2 must be expressed at a threshold level for E1^E4 to have an effect on E1/E2-mediated transcription. However, at levels of E1 and E2 which caused maximum levels of p97 activation, the expression of E1^E4 reduced the magnitude of E1/E2-mediated transactivation. In contrast, when E2 was expressed at levels that were too high relative to E1 to cause maximum transactivation, E1^E4 expression caused a relative increase in the level of transactivation. The differential effects of E1^E4 on transcription were shown to be independent of the G2 arrest function of E1^E4.

6.2 E1^E4 may change the nuclear level of E2

In this study we show that when E2 and E1^E4 are co-expressed in cells, the nuclear levels of E2 protein could be changed by the ability of E1^E4 to cause the relocation of E2 to the cytoplasm. The extent and frequency of cytoplasmic colocalisation between E2 and E1^E4 was found to increase with time of E2/E1^E4 co-expression. This resulted in a qualitative difference in nuclear E2 in E2/E1^E4 expressing cells as determined by immunofluorescence. This however, did not result in the complete loss of E2 from the nucleus, or in any significant alteration in the distribution of E1^E4. Thus, it is not surprising that E2 functions, which are critically dependent on its nuclear localisation are affected by E1^E4 co-expression with E2.

It has been speculated that the ratio of E1 to E2 proteins in the nucleus must be increased for genome amplification to occur in an HPV infection (Ozbun and Meyers, 1998a; Klumpp and Laimins, 1999). The level of E1 transcripts are thought to be

increased at the onset of genome amplification by the activation of the HPV late promoter, and also by an accumulation of E1-containing transcripts produced from the HPV early promoter (Ozbun and Meyers, 1998a; Klumpp and Laimins, 1999). It is also possible that the reduction in nuclear E2 by E1^E4, may also increase the ratio of E1 to E2 in the nucleus, and in this way enhance HPV genome amplification.

E2 acts to initiate HPV replication by targeting E1 to the origin of HPV replication (ori) (Sverdrup and Khan, 1994; Sverdrup and Khan, 1995; Gillitzer et al., 2000; Chen and Stenlund, 2001). However, it is imperative for the progression of replication that E2 is lost from the origin of replication to enable E1 to be assembled into an active complex (Lusky et al., 1994; Titolo et al., 1999). This situation is analogous to the loading of the cellular replication helicase (MCMs) onto DNA by cdc6, which is required to initiate cellular DNA replication (Bueno and Russell, 1992). Following the initiation of replication, cdc6 is sequestered in the cytoplasm to enable cellular replication to proceed (Petersen et al., 1999). Our data suggests that similarly, the sequestration of E2 in the cytoplasm by E1^E4 may be a mechanism of reducing E2-mediated inhibition of ori-dependent replication.

Coincident with an increase in cytoplasmic E2, in E1^E4 expressing cells, it was found that E1^E4 could increase E1/E2-mediated transactivation when E2 was expressed at inhibitory concentrations. This function of E1^E4 may allow an increase in transcription from the early promoter to occur at the onset of genome amplification. It has already been mentioned that an accumulation of E1 transcripts from the HPV early promoter coincides with an increase in genome amplification. In addition to E1 transcripts the levels of E6 and E7 transcripts from the HPV early promoter are also elevated in cells that are amplifying the HPV genome in productive infections (Durst et al., 1992b; Higgins et al., 1992; Ozbun and Meyers, 1998a). This increase in early transcripts coincides with an increase in E1, E2 and E1^E4 expression from the HPV late promoter (Higgins et al., 1992; Grassmann et al., 1996). It has been shown in this study and by others that high levels of E2 expression can inhibit the activity of the HPV early promoter (Cripe et al., 1987; Bernard et al., 1989; Stubenrauch et al., 1996; Stubenrauch et al., 1998). Yet despite this, an increase in E6, E7 and E1 transcription from the early promoter has been observed in productive HPV infections (Durst et al.,

1992b; Higgins et al., 1992; Grassmann et al., 1996; Ozbun and Meyers, 1998a). We speculate that a possible reduction in nuclear E2 levels in E1^E4 expressing cells may prevent the repression of the early promoter by high levels of E2 expression.

The cytoplasmic sequestration mechanism described here for the modulation of E2 by E1^E4 is similar to that reported for the regulation of cyclin B1 and E4-DBP by 16E1^E4 in other studies (Doorbar et al., 2000; Davy et al., 2005). The latter interactions have been shown to occur in the cytoplasm of cells in the intermediate layers of infected epithelium, which is the region of the epithelium in which it is suspected that the E2/E1^E4 interaction may occur *in vivo*. However, it was not clear from the experiments conducted in this study whether an association between E1^E4 and E2 does occur in HPV induced lesions. There is however, little doubt that 16E2 is expressed in the same cells as 16E1^E4 following the onset of genome amplification (Doorbar et al., 1997; Maitland et al., 1998; Stevenson et al., 2000; Middleton, 2003). This is because the E2 protein is essential for the initiation of vegetative viral DNA replication, and several reports have reported that the expression of E1^E4 coincides with genome amplification (Breitburd et al., 1987; Sverdrup and Khan, 1994; Sverdrup and Khan, 1995; Doorbar et al., 1997; Penrose and McBride, 2000; Peh et al., 2002). In support for the E2/E1^E4 interaction occurring *in vivo*, immuno-staining of HPV18-infected lesions by Dr. Woei Ling Peh (NIMR) gave interesting results, with 18E2 showing a specific cytoplasmic pattern which overlaps with 18E1^E4 in the region of the lesion where genome amplification is supported (Dr. Woei Ling Peh, *Personal Communication*).

6.3 E1^E4 can increase the levels of E2 protein

The protein and mRNA expression profiles that were produced by both the WT and mutant cassettes following 72 h of expression were analysed in the previous chapter. It was found that E1^E4 protein was only produced by the WT cassette, although both the WT and mutant cassettes produced equal levels of E1^E4 coding transcripts. This result was to be expected as two stop codons were present in the E4 ORF which inhibited the translation of the E1^E4 protein but not the transcription of the spliced ORF in the mutant cassette. The E1 protein could not be detected in cells transfected with either the WT or mutant cassettes. This was also expected, as the E1 protein is

only expressed at very low levels both *in vitro* and presumably *in vivo*, due to the presence of a strong splice donor site at the beginning of the E1 ORF (Remm et al., 1999; Wang et al., 2003). However, the quantitative analysis of E1 transcripts produced by both the WT and mutant cassettes showed that there was no statistical difference between the WT and mutant cassettes in t-test analysis at the 99 % confidence interval. Similarly, quantitative analysis of E2 transcripts produced by both the WT and mutant cassettes showed no statistical difference between the WT and mutant cassettes in t-test analysis at the 99 % confidence interval. However, the total level of E2 protein present in cells following transfection with the WT cassette was found to be approximately double that of the level produced by the mutant cassette following 72 h of transfection. Thus apart from the expected difference in E1^E4 protein expression, between the WT and mutant cassettes the other difference that was detected was in E2 protein levels. E2 turn-over is normally tightly regulated by the ubiquitin-proteasome pathway, which has been shown to result in the rapid loss of accumulated E2 in transfected cells (Penrose and McBride, 2000; Bellanger et al., 2001). We propose that the sequestration of E2 in the cytoplasm to E1^E4 bound structures may be able to reduce nuclear E2 levels, by decreasing the solubility of E2 protein. The tethering of E2 to E1^E4 in the cytoplasm may also increase the stability of the E2 protein, and in this way may serve as a mechanism of preventing the loss of E2, following high levels of E2 protein expression.

6.4 The E2/E1^E4 binding site

Two regions of 16E1^E4 have been shown to disrupt E2/E1^E4 binding in this study, the extreme C-terminus (84-92) and an amino acid stretch (36-41) in the highly charged central portion of the protein. In addition several E1^E4 deletion mutants in the central charged region of E1^E4 (31-68), were also found to reduce the robustness of the interaction between E1^E4 and E2. It is possible that the deletion of the C-terminal amino acids of E1^E4 may disrupt E2/E1^E4 binding either because they are directly involved in the E2/E1^E4 interaction, or because they effect the multimerisation of E1^E4 (Roberts et al., 1997; Wang et al., 2004). Similarly, deletions in the interior of the E1^E4 primary sequence may disrupt E2/E1^E4 binding either because they are critical for the E2/E1^E4 interaction, or alternatively because these deletions may effect the structure of the E1^E4 protein.

Further experiments are needed to test these possibilities, however characterisation of the E2/E1^E4 interaction suggests that the binding of E2 to E1^E4 involves hydrophobic contacts. This makes it more likely that the C-terminal E1^E4 residues are involved in E2 binding than the central residues because the former region is uncharged. Unpublished data provided by Helena Sterlinko Grm (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy) also supports the evidence presented here that the E2/E1^E4 interaction is hydrophobic in nature, as their data suggest that the amino acids 322-339 of 16E2 are critical for E1^E4 binding. This region of E2 incorporates the disordered loop region of the E2 C-terminus (which it is thought becomes ordered upon DNA binding), the β_3 strand (involved in E2 dimerisation), and residues of the first turn of the DNA recognition α_2 helix (Figure 1.5a; (Hegde and Androphy, 1998)). It is interesting that the hydrophobic C-terminal region of E2 is β-stranded and the hydrophobic C-terminal region of E1^E4 is also thought to be β-stranded (Hegde and Androphy, 1998; Dr. Pauline McIntosh, NIMR, *Personal Communication*). Thus it is possible that the E2/E1^E4 interaction may result in the formation of a β-sheet structure between the two proteins, which may affect the ability of the E2 protein to multimerise or to associate with DNA.

The implication of the C-terminus of E2 in E1^E4 binding also raises the possibility that in addition to binding to the full length E2 protein, E1^E4 may bind and sequester E2 splice variants such as E2C in the cytoplasm. The E2C protein lacks the N-terminal domain of E2, and has been shown experimentally to inhibit transcription (Doorbar et al., 1990; Bouvard et al., 1994). Transcripts encoding E2C were found to be produced by both the WT and mutant cassettes in this study, and previously by the HPV16 genome (Doorbar et al., 1990). Thus it is possible that E1^E4 could also sequester the E2C protein in the cytoplasm as well as E2, given that the E1^E4 binding site on E2 is thought to be located in the C-terminal domain of the protein, which could effect the activity of the early promoter.

6.5 E1^E4 interaction with proteins expressed in an HPV infection

In addition to binding to E2, E1^E4 was also found to be able to associate with the E7, L1 and L2 proteins of HPV16, and also potentially nine other proteins that are expressed in epithelial cells. E7, E2, L1 and L2 proteins are all thought to be co-

expressed with E1^E4 in the intermediate and upper layers of the epithelium in various combinations (Doorbar et al., 1997; Maitland et al., 1998; Di Lonardo et al., 2001; Middleton, 2003). Thus it is possible that these novel interactions may be relevant in the context of the late stages of the virus life cycle. The cellular proteins that associated with E1^E4 in this study were not identified. However, it is possible that the association of E1^E4 with these yet unknown epithelial proteins may be important in modulating E1^E4 function *in vivo*. Both HPV and cellular proteins that bound to E1^E4 displayed differences in their binding preferences for the differentially phosphorylated forms of E1^E4. These differences in E1^E4 binding specificity may provide a way of regulating E1^E4 binding partners during a productive infection.

The expression patterns of different cleaved and phosphorylated forms of HPV1 E1^E4 proteins change with differentiation, however, it is uncertain whether similar changes in E1^E4 expression occur in HPV16 infections (Breitburd et al., 1987). However, differences in the intracellular distribution of the N-terminally cleaved and the different phosphorylated forms of genital HPV E1^E4 proteins have been described (Doorbar et al., 1997; Bryan et al., 2000). These subtle variations in the expression patterns of the different forms of E1^E4 may contribute to the regulation of the association of E1^E4 to different proteins in the HPV life cycle.

E1^E4 was found to be able to change the intracellular distribution of E2. It remains to be established whether E1^E4 can alter the distribution and/or the function of the E7 protein or vice-versa. This study and others have shown that when HPV proteins that have been shown to associate *in vitro* are co-expressed, the distribution in the cell of one of the HPV proteins often changes. This is exemplified by the co-expression of E1 and E2, L2 and E2, L1 and L2 and in this study for E2 and E1^E4 (Day et al., 1998; Swindle et al., 1999; Florin et al., 2002). HPV16 E7 can be considered as a nuclear protein, and accumulates at nucleoli when expressed in cell culture. However, when E7 expressing cells are held in the G2 phase of the cell cycle E7 has been found to relocate to the cytoplasm (Zatsepina et al., 1997). This is intriguing given that a function that is common to several E1^E4 proteins including 16E1^E4, is the ability to cause G2/M arrest (Davy et al., 2002; Nakahara et al., 2002; Knight et al., 2004). Thus, it is possible that cytoplasmic E7 alone, or in combination with E1^E4 may play

a different role in the virus life cycle to the roles that have been documented for nuclear E7 in the promotion of S-phase.

Preliminary observations by Dr. Woei Ling Peh (NIMR) suggest that when E1^E4 is co-expressed with the capsid proteins L1 and L2 in monolayer culture, E1^E4 can adopt the expression patterns assumed by the viral capsid proteins in a minority of cells (Peh et al., 2004b). This is in contrast to what was found when E2 and E1^E4 proteins were expressed together, as in this case E2 was found to conform to the E1^E4 expression pattern in the majority of cells. It is interesting that E1^E4 can affect the expression patterns of E2, but that L1 and L2 can affect the expression patterns of E1^E4 in cell culture. This may allow the function of E1^E4 in the virus life cycle to change with differentiation. Thus, it is possible that E1^E4 may enhance viral genome amplification and early gene transcription in the intermediate layers of the epithelium by regulating nuclear E2 levels, but in the superficial layers of a productive infection E1^E4 may cooperate with L1, L2 and maybe even E2 for the completion of the HPV life cycle.

It was intriguing to find that HPV16 E2 and L2 which were shown in this study and by others, to interact *in vitro* can also associate with the E1^E4 protein (Okoye et al., 2005). The interaction between E2 and L2 is thought to facilitate packaging of the replicated viral genomes into particles (Day et al., 1998; Zhao et al., 2000). It is possible that E1^E4 may also participate in this process by forming a complex with E2, L2 and L1. The association of E1^E4 with L1 and L2 may even increase the stability of HPV virions. This may allow virus particles to survive in the environment, in a manner similar to that in which baculovirus virions are protected from adverse effects by association with polyhedrin (Minion et al., 1979). In fact, such a function for E1^E4 was first suggested following its original discovery by the Gallimore lab in 1986 (Doorbar et al., 1986).

6.6 Conclusions

- E1^E4 was shown to bind to several viral (direct binding) and cellular proteins in this study. It was also shown that these proteins associated discriminately with the different phosphorylated forms of E1^E4. Together this suggests that E1^E4 may be able to associate with numerous HPV and cellular proteins during the virus life cycle, and that the binding of E1^E4 to these different proteins could be regulated by the phosphorylation status of the protein.

- From work carried out towards the end of the project it was shown that E1^E4 was able to increase the level of E1/E2-mediated HPV origin-dependent replication, and also to modulate the levels of E1/E2-mediated early promoter activation i.e. E1^E4 could reduce the level of HPV16 early promoter (p97) activity when E2 was expressed at optimised levels, but could increase the level of p97 activity when E2 was expressed at inhibitory levels. However, when E2 and E1^E4 were expressed together with E1 at ratios that mimicked those seen *in vivo* following the activation of the differentiation-dependent promoter, E1^E4 consistently enhanced p97 activity. The ability of E1^E4 to decrease the inhibitory effect of E2 on transactivation was found to increase with the cytoplasmic accumulation of E2. We propose that E1^E4 may modulate the nuclear activities E2 by reducing the levels of nuclear E2.

- E1^E4 was also found to increase E2 levels at late time points following transfection. This effect of E1^E4 may also contribute to the increased levels of E1/E2-mediated transcription and replication that were observed in transient assays. We speculate that the association of E2 and E1^E4 in the cytoplasm of cells may stabilise E2.

6.7 Evaluation of project aims

6.7.1 Identification of novel HPV16 E1^E4 binding partners

The primary objective of this thesis was to identify proteins that could associate with 16E1^E4 either directly or indirectly *in vitro*. HPV16 E1^E4 specific co-immunoprecipation experiments suggested that E1^E4 could associate with at least nine cellular proteins, however, the identities of these proteins were not revealed in this study. In addition to this, 16E1^E4 was found to associate with several viral proteins via direct interactions. The novel interactions identified were between E1^E4 and E2, E1^E4 and E7, E1^E4 and L1 and also between E1^E4 and L2.

6.7.2 Identification of the intracellular localisation of E1^E4 protein complexes

It was envisaged E1^E4 could affect the intracellular distribution of possible novel interacting proteins, and so ideally the cellular localisation of all novel E1^E4 binding proteins were to be investigated. However, due to time limitations it was only feasible to investigate one of the interactions identified thoroughly. The novel interaction that was chosen as the focus of this study was between 16E2 and 16E1^E4. It was found that the co-expression of both of these proteins in monolayer culture resulted in the increased accumulation of E2 protein in the cytoplasm, but only rarely did this cause the complete loss of nuclear E2. Cytoplasmic E2 was found to conform to the E1^E4 expression pattern in some cells when co-expressed with E1^E4 i.e. E2 displayed either filamentous, perinuclear or punctate patterns in the cytoplasm similar to those produced by E1^E4. Cytoplasmic E2 was seen in the minority of E2 expressing cells in the absence of E1^E4, but in the absence of E1^E4, cytoplasmic E2 was not found to give either filamentous, perinuclear or punctate distributions.

6.7.3 Investigation of the functional significance of HPV protein interactions with E1^E4

From the onset of this study there was the intent to investigate the functional significance of novel E1^E4-HPV protein interactions if they were identified. Despite the identification of four novel E1^E4/HPV protein interactions, because of time limitations the functional significance of only the E2/E1^E4 interaction was addressed

in this study. The major roles of E2 in the HPV life cycle are in the regulation of early gene transcription and viral genome replication. Thus, the effect of E1^E4 on these functions of E2 were investigated.

It was found that E1^E4 could increase, reduce or have no effect on E2-dependent transactivation of the HPV16 early promoter depending on the ratios and levels of the two proteins. However, when E2 and E1^E4 were expressed at controlled levels from an early HPV16 gene cassette, E1^E4 was found to reproducibly increase E2-dependent transcription from the early promoter provided both proteins were expressed above a threshold level. E1^E4 was also found to increase ori-dependent replication in transient assays.

6.7.4 To separate any novel effects of E1^E4 on the HPV life cycle from its G2-arrest function

To attribute any effects seen on any of the HPV life cycle functions that were to be investigated from those caused by E1^E4-mediated G2 arrest. Initially it was envisaged that the use of a characterised G2-arrest defective mutant would be useful in separating the influence of the G2 arrest from the possible function(s) of any novel E1^E4 protein interactions that was being investigated. This mutant was used to successfully eliminate the contribution of E1^E4-mediated G2 arrest on E2-dependent transcription. However, the G2-arrest defective mutant was not useful for eliminating the effect of E1^E4-mediated G2 arrest on replication, as replication assays were performed using an E1/E2/E4 and E1/E2 cassette expression vectors which were incompatible with the G2-arrest defective mutant expression vector. Thus, the increase in replication seen in the presence of E1^E4 could at least in part be due to E1^E4-mediated G2 arrest.

6.8 Future Work

6.8.1 Identify cellular E1^E4 binding proteins

Nine cellular proteins were found to associate with E1^E4 in co-immunoprecipation experiments. Some of these were found specifically to associate with soluble E1^E4 (unphosphorylated E1^E4) and some with insoluble E1^E4 (both phosphorylated and unphosphorylated E1^E4). It would be interesting to identify these proteins and also to investigate their potential roles in the virus life cycle.

6.8.2 Investigate the E1^E4/E7 interaction

The initial screening of E1^E4 against other HPV16 proteins revealed that E2, E7, L1 and L2 could associate with E1^E4 directly. The E2/E1^E4 interaction was characterized in this study and its functional significance was investigated. Similarly, the significance of the E1^E4 interaction with the viral capsid proteins is currently being investigated by Dr. Woei Ling Peh (NIMR). However, the E1^E4/E7 interaction has not been investigated further. It would be interesting to see if the G2 arrest function of E1^E4 promotes the interaction of E7 and E1^E4 in cells, as speculated in Section 7.4. And if this is the case another curiosity that could be tested is the effect of the association between E7 and E1^E4 on the phosphorylation status of E7 or E7-associated proteins, given that E1^E4 has been shown to associate with an active cyclin B1-cdk 1 complex in the cytoplasm of cells (Davy et al., 2005).

6.8.3 Identify the critical amino acids involved in the E2/E1^E4 interaction

Two regions of E1^E4 were identified in this study that the deletion of which could prevent E1^E4 from binding to E2. However, it was not conclusively shown that that these regions contained the key amino acids responsible for making contact with E2. Alanine scanning of the regions of E1^E4 that were found to disrupt the E2/E1^E4 interaction should be a feasible way of identifying the critical E1^E4 residues that are involved in binding to E2. A similar approach could also be employed to pin-point the essential amino acids in E2 involved in binding E1^E4. These E2 and E1^E4 mutants could then be used in functional assays like those that have been described in this study. This would enable the effects of E2 and E1^E4 binding on replication and transcription to be addressed directly.

6.8.4 Test whether E2/E1^E4 proteins from other papillomaviruses interact

Conserved interactions of PV proteins between PV types are likely to be essential in the virus life cycle, whereas niche interactions may be important in providing specific characteristics to sub-groups of PVs. In this study the E2/E1^E4 interaction was found to occur between E2 and E1^E4 proteins from two high-risk HPVs. It would be interesting to ascertain whether the ability of E2 and E1^E4 proteins of HPV16 and HPV18 to interact could be extended to other PV types.

6.8.5 Quantitative assessment of nuclear E2 levels in the absence and presence of E1^E4

In this study the frequency of cytoplasmic E2 accumulation in the presence and in the absence of E1^E4 protein expression was assessed. Coincidently a qualitative difference in nuclear E2 levels was noted in E1^E4 expressing cells, however actual levels of nuclear E2 in the presence and absence of E1^E4 could not convincingly be shown by nuclear/cytoplasmic fractionation due to the innate difficulties associated with the fractionation of E1^E4 expressing cells (Davy et al., 2005). The levels of nuclear E2 are critical to its function so it is important to be able to compare the levels of nuclear E2 in cells which are, and are not, expressing E1^E4. An alternative approach to measure nuclear E2 levels would be to use quantitative immunofluorescence confocal microscopy.

6.8.6 Investigation of other factors that influence the E2/E1^E4 interaction

E2 and E1^E4 proteins were shown at best to colocalise in the cytoplasm of 81 % transfected cells. This suggests that other factors apart from the ability of the two proteins to bind to each other may influence the interaction. It is possible that the interaction between E2 and E1^E4 may be facilitated by differentiation, given that in this study the cell death process seemed to increase the frequency of colocalisation. Thus, it would be interesting to study the immunofluorescence patterns of E2 and E1^E4 in differentiating cells.

6.8.7 Establish whether E2 and E1^E4 proteins colocalise *in vivo*

In this study double-staining of E2 and E1^E4 proteins in HPV16-infected lesions and raft sections was tested. However, the results of these experiments were inconclusive, because the background staining of the E2 antibodies that were used was very high. This suggested that the staining conditions that were employed were not optimal for the detection of E2. It is important to ascertain whether the interaction between E2 and E1^E4 occurs or not *in vivo*. Thus optimization of the staining protocol using available or even possibly new E2 antibodies is required. Another viable way of addressing this question is immuno-staining of HPV18 lesions as an interaction between 18E2 and 18E1^E4 has also been shown in this study.

6.8.8 Investigate the repressive effect of E1^E4 on the LCR

E1^E4 alone was shown to be able to completely repress transcription from the HPV16 LCR in cell-based transcription assays. It would be interesting to test whether E1^E4 could repress E6 and E7 transcription in W12e cells as well. This is because this cell type contains episomal copies of the HPV16 genome packaged with histones. The chromatin conformation of the promoter and the LCR of episomal HPV16 genomes have been shown to restrict the effect of E2 on transcription (Bechtold et al., 2003). Thus it would be interesting to see if E1^E4 could effect the activity of the p97 promoter when it is packaged in a state comparable to that in basal cells.

6.8.9 Test the effect of the E2/E1^E4 association on the half life of E2

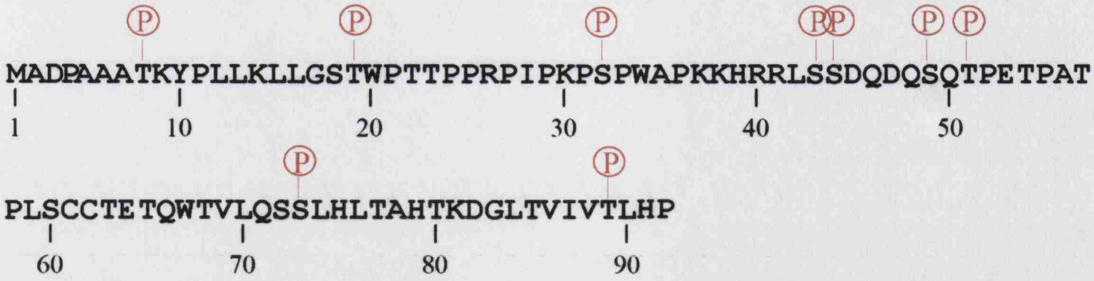
It was speculated in this study that the cytoplasmic sequestration of E2 by E1^E4 may prevent the complete loss of E2 in E1^E4 positive cells at late time points by E2 stabilization in E1^E4 expressing cells. It was hypothesized that this would result in a greater number of transfected cells still expressing E2 following transfection for 72 h with the WT cassette compared to the mutant cassette. To test this, cells transfected with the WT and mutant cassette (i.e. eGFP positive) could be separated and the number of cells that were E2 positive in both samples could be compared.

6.8.10 Detection of E1 and E2C protein from the WT and mutant cassettes

The E1 and E2C ORFs were both shown to be transcribed by the WT and mutant cassettes in this study, but both proteins were only thought to be expressed at low

levels. Difficulties arose in this study in trying to identify the E1 protein by Western blotting. To circumvent this problem in the future it may be possible to assess the relative levels of E1 produced by the cassettes using an *in vitro* transcription/translation system. In this system the proteins could be labelled by the incorporation of radioactive amino acids which could mean that these proteins could be detected by more sensitive methods than Western blotting. This method could also be used to detect E2C or alternatively Western blotting of WT and mutant cassette transfected cell extracts with an E2C specific antibody could also be tried.

a



b

Threonine predictions

Pos	Context (v)	Score	Pred
8	PAAATKYPL	0.875	*T*
19	LLGSTWPTT	0.017	.
22	STWPTTPPR	0.155	.
23	TWPTTPPRP	0.768	*T*
51	DQSQTPETP	0.655	*T*
54	QTPETPATP	0.248	.
57	ETPATPLSC	0.265	.
63	LSCCTETQW	0.013	.
65	CCTETQWTV	0.054	.
68	ETQWTVLQS	0.053	.
77	SLHLTAHTK	0.033	.
80	LTAHTKDGL	0.228	.
85	KDGLTVIVT	0.156	.
89	TVIVTLHP-	0.042	.

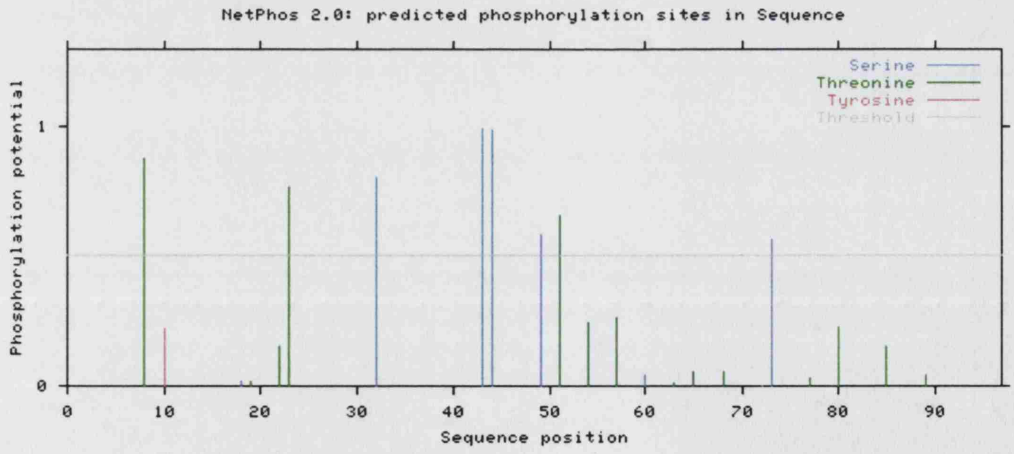
Serine predictions

Pos	Context (v)	Score	Pred
18	KLLGSTWPT	0.016	.
32	IPKPSPWAP	0.806	*S*
43	HRRLSSDQD	0.995	*S*
44	RRLSSDQDQ	0.990	*S*
49	DQDQSQTPE	0.582	*S*
60	ATPLSCCTE	0.041	.
72	TVLQSSLHL	0.003	.
73	VLQSSLHLT	0.566	*S*

Tyrosine predictions

Pos	Context (v)	Score	Pred
10	AATKYPLLK	0.218	.

c



The output from NetPhos http://www.cbs.dtu.dk/services/NetPhos/

(a) The amino acid sequence of 16E1^E4. Residues having a phosphorylation prediction score above a threshold value are indicated. (b) The predictions for each type of residue (Ser, Thr or Tyr). In each table is shown the position of the residue being analyzed, the sequence context (shown as a 9-residue sequence centered on the residue being analyzed), the output score (value in the range [0.000-1.000]) and the assigment (scores above the threshold of 0.500 are assigned as '*S*', '*T*', or '*Y*'). (c) A graph illustrating the predictions

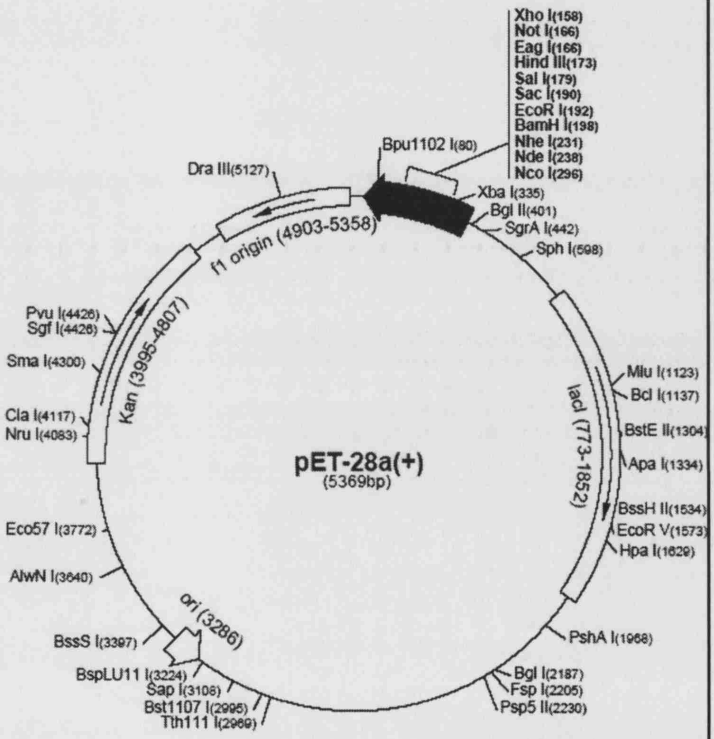
pET-28a-c(+) Vectors

	Cat. No.
pET-28a DNA	69864-3
pET-28b DNA	69865-3
pET-28c DNA	69866-3

The pET-28a-c(+) vectors carry an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

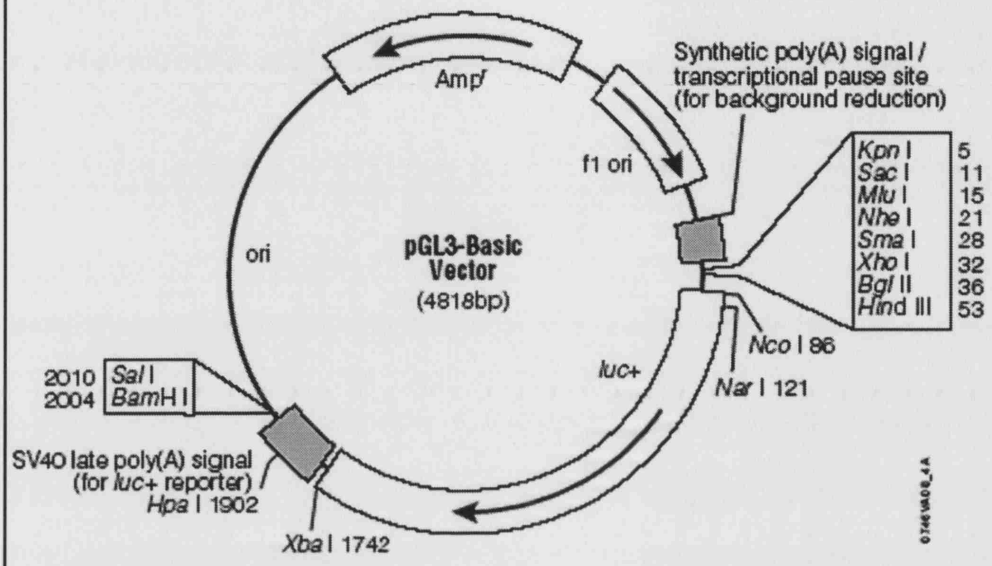
The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.



pET-28a-c(+) cloning/expression region

Plasmid map was obtained from
http://www.emdbiosciences.com/docs/docs/PROT/TB074.pdf

pGL3 Vector Maps and Sequence Reference Points

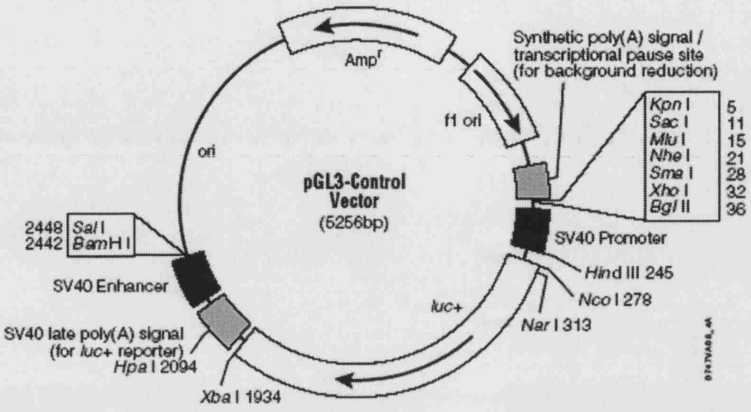


pGL3-Basic Vector circle map. Additional description: *luc+*, cDNA encoding the modified firefly luciferase; Amp^r, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E. coli*. Arrows within *luc+* and the Amp^r gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis.

pGL3-Basic Vector Sequence Reference Points:

SV40 Promoter	(none)
SV40 Enhancer	(none)
Multiple cloning region	1–58
Luciferase gene (*luc+*)	88–1740
GLprimer2 binding site	89–111
SV40 late poly(A) signal	1772–1993
RVprimer4 binding site	2080–2061
*Col*E1-derived plasmid replication origin	2318
β-lactamase gene (Amp^r)	3080–3940
f1 origin	4072–4527
Synthetic poly(A) signal	4658–4811
RVprimer3 binding site	4760–4779

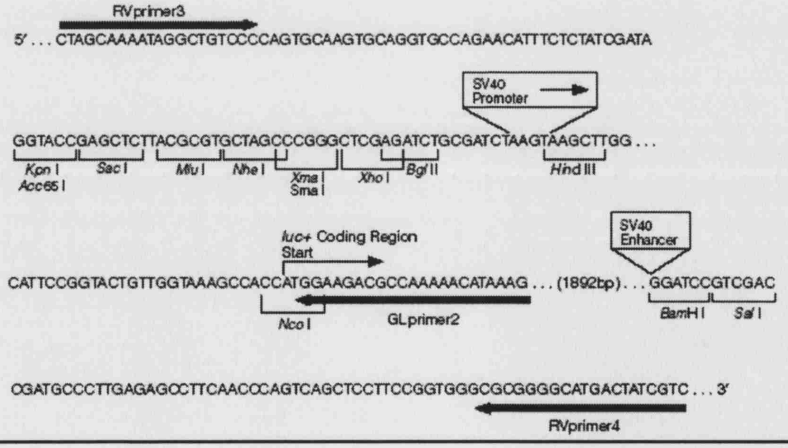
Plasmid map was obtained from http://www.promega.com/tbs/tm033/tm033.pdf



pGL3-Control Vector circle map. Additional description: *luc+*, cDNA encoding the modified firefly luciferase; Amp^r, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E. coli*. Arrows within *luc+* and the Amp^r gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis.

pGL3-Control Vector Sequence Reference Points:

Multiple cloning region	1–41
SV40 Promoter	48–250
Luciferase gene (*luc+*)	280–1932
GLprimer2 binding site	281–303
SV40 late poly(A) signal	1964–2185
SV40 Enhancer	2205–2441
RVprimer4 binding site	2499–2518
*Col*E 1-derived plasmid replication origin	2756
β-lactamase gene (Amp^r)	3518–4378
f1 origin	4510–4965
Synthetic poly(A) signal	5096–5249
RVprimer3 binding site	5198–5217



Plasmid map was obtained from http://www.promega.com/tbs/tm033/tm033.pdf

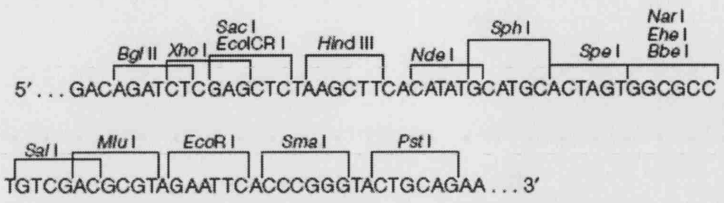
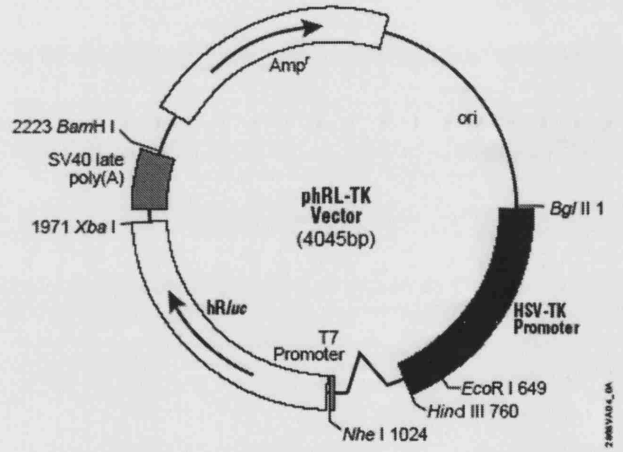


Figure 4. Multiple cloning region of the phRL-null Vector.



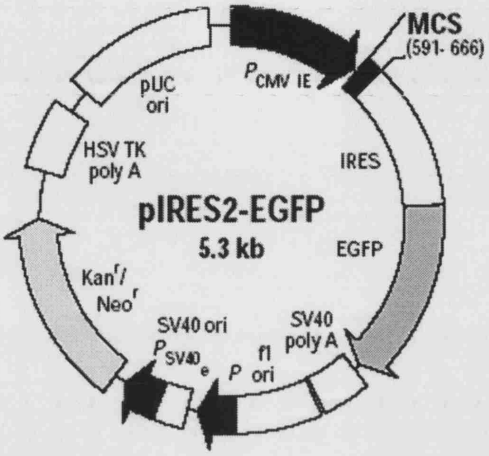
The phRL-TK Vector circle map. Additional description: –^–, position of intron; hR*luc*, synthetic cDNA sequence encoding the *Renilla* luciferase enzyme; Amp^r, gene conferring ampicillin resistance in *E. coli;* ori, origin of plasmid replication in *E. coli.* Arrows within the hR*luc* and Amp^r gene indicate the direction of transcription.

phRL-TK Vector Sequence Reference Points:

HSV-TK promoter	7–759
Chimeric intron	826–962
T7 RNA polymerase promoter (–17 to +2)	1006–1024
T7 promoter transcription start site	1023
hR*luc* reporter gene	1034–1969
SV40 late poly(A) region	1991–2212
β-lactamase (Amp^r) coding region	2359–3219

Plasmid map was obtained from http://www.promega.com/tbs/tm237/tm237.pdf

pIRES2-EGFP Vector Information



591 601 611 621 631 641 651 661

GCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCC...IRES

Nhe I *Eco47* III *Bgl* II *Xho* I *Sac* I *Ecl136* II *Hind* III *EcoR* I *Pst* I *Sal* I *Acc* I *Kpn* I *Asp718* I *Sac* II *Apa* I *Bsp120* I *Xma* I *Sma* I *Bam*H I

Restriction Map and Multiple Cloning Site (MCS) of pIRES2-EGFP Vector. Unique restriction sites are in bold. Note that the *Eco*47 III site has not been confirmed in the final construct.

Plasmid map was obtained from
http://www.clontech.com/clontech/techinfo/vectors/vectorsF-I/pdf/PT3267-5.pdf

Appendix III : Cloning Primers

Name	Sequence 5'-3'	Length (bases)	Position	Vector
LCR F	CTT GAT **GCT AGC** C_{7031}AG ACC TAG ATC AGT TTC CTT TAG G_{7007}	37	Blue is NheI site, 24 bases match LCR, nt 7007-7031 (Accession no. AF125673)	pGL3.Basic (Promega)
LCR R	CTT GAT **AGA TCT** T_{85}GC AGT TCT CTT TTG GTG C_{103}	31	Blue is BglII site, 19 bases match LCR, nt 85 to 103 (Accession no. AF125673)	pGL3.Basic (Promega)
E1^E4 F	CTT GAT **CCA** $_1$ **TGG** CTG ATC CTG CAG CAG CAA C_{23}	31	Blue is NcoI site, 21 bases match E1^E4 gene, nt 1 to 23	pET-28(b)+ (Novagen)
E1^E4 R	GTG ACT **CTC GAG** T_{279}GG GTG TAG TGT TAC TAT TAC_{258}	33	Blue is XhoI site, 21 bases match E1^E4, nt 258 to 279	pET-28(b)+ (Novagen)
E1^E4 F Mut 1	CTT GAT **CC ATG** G_{13}CA GCA ACG AAG TAT CCT CTC_{33}	32	Blue is NcoI site, 21 bases match E1^E4, nt 13 to 33	pET-28(b)+ (Novagen)
E1^E4 R Mut 13	GTG ACT **CTC GAG** T_{279}GG GTG TAG TGT_{268} TCC GTC C	31	Blue is XhoI site, 19 bases match E1^E4, nt 268 to 279 and	pET-28(b)+ (Novagen)
E1^E4 R Mut 14	GTG ACT **CTC GAG** AGT TAA TCC GTC CTT TGT GTG	33	Blue is XhoI site, 21 bases match E1^E4, nt	pET-28(b)+ (Novagen)
Cassette F	ACG T**GA ATT C**TA ATC TAC C ATG_{865} GCT GAT CCT GCA GGT ACC AAT_{888}	43	Blue is EcoRI site, 24 bases match E1, nt 865 to 888 (Accession no. AF125673)	pIRES2. eGFP (Invitrogen)
Cassette R	ACG TTC TAG ACG C**GG ATC C** T_{3852}CA TAT AGA CAT AAA TCC AGT AGA_{3829}	43	Blue is BamHI site, 24 bases match E2, nt 3852 to 3829 (Accession no. AF125673)	pIRES2. eGFP (Invitrogen)

Appendix IV : Expression Vectors

Expression construct	Source or reference	Additional information
rAd.16E1^E4	(Doorbar et al., 2000)	
rAd.16E2	(Fournier et al., 1999)	
rAd.βGAL	(Fournier et al., 1999)	
pMV11.16E1	Dr. Ken Raj, NIMR	
pMV11.16E2	Dr. Ken Raj, NIMR	
pMV11.16E1^E4	(Davy et al., 2002)	
pMV11.T22A, T23A 16E1^E4	(Davy et al., 2005)	
pGBT9.M1-M13	(Doorbar et al., 2000)	Mutagenesis was originally performed by Roberts *et al* (Roberts et al., 1997)
pMAL.16E1^E4	(Doorbar et al., 1997)	
pGEX.16E1	(Hibma et al., 1995)	
pGEX.16E2	(Hibma et al., 1995)	
pGEX.16E1^E4	(Doorbar et al., 1992)	
pGEX.16E5	Dr. Lawrence Banks, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy	
pGEX.16E6	(Keen et al., 1994)	
pGEX.16E7	(Ciccolini et al., 1994)	
pGEX.16L1	(Zhou et al., 1991)	
pGEX.16L2	Dr. Toshi Sasagawa, Kanazawa University, Ishikawa, Japan.	
pGEX.18E2	Dr. Françoise Thierry, Pasteur Institute, Paris, France.	
pTK-luc pTKM-luc	Roland Sahli, Institute of Microbiology, Lausanne, Switzerland.	Originally the thymidine kinase promoter was cloned downstream of six E2 BSs (ACCG AAAA CGGT) by Thierry *et al* in pCAT (Thierry et al., 1990).
pCMV.E1	(Wang et al., 2003)	Codon-optimised for expression in eukaryotic cells.
pCR4-TOPO.βactin	Dr. Sebastien Viatte, Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland	

Appendix V RT-PCR Primers

Name	Sequence 5'-3'	Length (bases)	Position
E1 F (qRT-PCR)	CAA AGT AGA AGA GCT GCA AAA AGG AGA TT	29	1216-1241 (Accession no. AF125673)
E1 R (qRT-PCR)	TAA CAT CTG CTG CTG AGT TTC CAC TTC A	28	1275-1299 (Accession no. AF125673)
E2 F (qRT-PCR)	TGA TAG TAC AGA CCT ACG TGA CCA TAT AGA	30	2818-2847 (Accession no. AF125673)
E2 R (qRT-PCR)	CCC ATT TCT CTG GCC TTG TAA T	22	2883-2904 (Accession no. AF125673)
β-actin F (qRT-PCR)	TGG GCA TGG GTC AGA AGG AT	20	207-226 (Accession no. NM_001101)
β-actin R (qRT-PCR)	CGG CCA GAG GCG TAC AGG GA	20	494-513 (Accession no. NM_001101)
E1 F	GAA GTG GGG GAG AGG GTG TTA GTG	24	1387-1407 (Accession no. AF125673)
E1 R	CAT TCT GGC GTG TCT CCA TAC ACT TC	26	1854-1879 (Accession no. AF125673)
E2 F	GAT CCT GCA G/AC GTG GTC CA	20	871-880/2710-2719 (Accession no. AF125673)
E2 R	GCA TAT GTC TCC ATC AAA CTG C	21	3111-3132 (Accession no. AF125673)
E2C F	GCA GAT GTT ACA GG/C AGC AAC GAA G	25	1289-1302/3358-3367 (Accession no. AF125673)
E2C R	CAT TTT AAA GTA TTA GCA TCA CCT TTT AAA TG	31	3622-3653 (Accession no. AF125673)

Appendix VI : Statistical analysis

E1 transcripts one-tail T-test

Ho: No significant difference in E1 transcript levels produced by the WT and mutant cassettes.

WT cassette (Δ Ct)	Mutant cassette (Δ Ct)
0.007562	0.006738
0.004908	0.007294
0.007783	0.004566
0.004872	0.006005
----------	0.004501

Two-sample variance F-test to check data is normally distributed

	WT cassette (Δ Ct)	Mutant cassette (Δ Ct)
Mean	0.006281	0.005821
Variance	2.59E-06	1.59E-06
Observations	4	5
Degrees of freedom	3	4
F calculated	1.627748	
P(F<=f) one-tail	0.317141	
F critical one-tail	6.591392	

$\mathbf{F_{critical} > F_{calculated}}$

Therefore, the ratio of the variances is not significantly different from 1. This implies with 95 % confidence that there is no difference the variances between WT and mutant cassette samples.

Results of One-tail T-test

	WT cassette (Δ Ct)	Mutant cassette (Δ Ct)
Mean	0.006281	0.005821
Variance	2.59E-06	1.59E-06
Observations	4	5
Pooled Variance	2.02E-06	
Hypothesized Mean Difference	0	
Degrees of freedom	7	
t calculated	0.482889	
t critical one-tail	2.998	

$T_{critical} > T_{calculated}$

Therefore, the null hypothesis of no significant difference in E1 transcript levels produced by the WT and mutant cassette between WT and mutant cassettes is accepted with 99 % confidence.

E2 transcripts one-tail T-test

Ho: No significant difference in E1 transcript levels produced by the WT and mutant cassettes.

WT cassette (Δ Ct)	Mutant cassette (Δ Ct)
0.000346	0.000315
0.000364	0.0003
0.000285	0.000291
0.000275	0.000238

Two-sample variance F-test to check data is normally distributed

	WT cassette (Δ Ct)	Mutant cassette (Δ Ct)
Mean	0.000318	0.000286
Variance	1.95E-09	1.12E-09
Observations	4	4
Degrees of freedom	3	3
F calculated	1.73848	
P(F<=f) one-tail	0.330428	
F critical one-tail	9.276619	

$\mathbf{F_{critical} > F_{calculated}}$

Therefore, the ratio of the variances is not significantly different from 1. This implies with 95 % confidence that there is no difference the variances between WT and mutant cassette samples.

Results of One-tail T-test

	WT cassette (Δ Ct)	Mutant cassette (Δ Ct)
Mean	0.000318	0.000286
Variance	1.95E-09	1.12E-09
Observations	4	4
Pooled Variance	1.54E-09	
Hypothesized Mean Difference	0	
Degrees of freedom	6	
t calculated	1.135085	
t critical one-tail	3.143	

$\mathbf{T_{critical} > T_{calculated}}$

Therefore, the null hypothesis of no significant difference in E2 transcript levels produced by the WT and mutant cassette between WT and mutant cassettes is accepted with 99 % confidence.

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